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CANCER RESEARCH

VOLUME 9 APRIL 1949 NUMBER 4

Adrenal Medullary Tumors (Pheochromocytomas) in Mice*

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Few spontaneous tumors of the adrenal medulla in laboratory animals have been reported. Slye, Holmes and Wells (8) described three mesotheliomas in the adrenal cortices of 33,000 mice. Adrenal paragangliomas were observed in three cows, one sheep and five horses (6). Hueper and Martin (4) found a neoplasm with the characteristics of a ganglioneuroma in the adrenal medulla of a castrated male rat among a series of thirty that had been maintained twelve months on a diet deficient in vitamin E. A medullary pheochromocytoma has been observed in an eighteen month old hamster (7), and in a twenty-one month old female mouse (5). A positive chromaffin reaction was not obtained in the latter tissue although the cellular characteristics were those of a pheochromocytoma. A medullary tumor reported by Whitehead (12) was not classified. The cells were morphologically comparable to those in the normal medulla but this tumor as well as that reported by Lowenthal (5) had a negative chromaffin reaction. Several investigations concerned with neoplasms in castrated mice of inbred strains have reported no demonstrable disturbances in the adrenal medulla (3, 9, 10, 12). More recent studies on physiologically or surgically castrated mice have indicated the presence of medullary tumors with the characteristics of pheochromocytomas.

* This work was supported by grants from The Jane Coffin Childs Memorial Fund for Medical Research, The Anna Fuller Fund and the U.S. Public Health Service.

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MATERIALS AND PROCEDURES

One hundred thirty-four mice of the BRS stock were castrated when 4 to 8 weeks of age and maintained on a commercial diet (Purina Fox Chow) and water *ad libitum* in an air conditioned room (temperature 75° ± 2° and humidity 50 per cent). Eighty-one mice from five strains and two hybrid groups were irradiated with x-rays beginning when 30 to 40 days of age (1½ minutes a day for 12 days—total dose of approximately 750 roentgen units—125 KV, 5 ma., 30 cm. target distance and 3 mm. of aluminum). They were subsequently maintained on the same diet as were the animals above. Nineteen animals of the later group received no further treatment, thirteen received either pellets of stilbestrol or subcutaneous injections of estradiol benzoate (16.6 μg. per week) for a period of three months beginning shortly after irradiation. Ten mice received 1 mgm. of progesterone weekly and 19 received 1.25 mgm. of testosterone propionate weekly beginning about one month after irradiation and continuing through life. Seventeen mice received a gonadotropic extract prepared from pregnant mare's serum and extending from the second to the fifth month after irradiation.

The mice were killed when their condition indicated the imminence of death or when tumors developed. Several endocrine glands, including the adrenal glands, and other tissues, were fixed in Bouin's fluid. Some of the sectioned adrenal glands were "post-chromed" on the slides and all were stained in hematoxylin and triosin.

OBSERVATIONS

Only one of the adrenal tumors in mice of the BRS strain enlarged the adrenal glands appreciably. The other three tumors or lesions were too small to detect at necropsy and were diagnosed only upon histological examination. The enlarged gland was yellowish, nodular and measured approximately 5 mm. in diameter. The lesions were unilateral in 2 mice.

Microscopic examination revealed thinner adrenal cortices in the mice bearing the tumors. Only capsular tissue remained around part of the tumorous gland of the mouse with the largest lesion and the remainder of the cortex was thin (Fig. 1). The cells were polyhedral, had poorly defined boundaries and were arranged in trabeculae or strands separated by vascular sinusoids that were, in some areas, distended. The cytoplasm of the tumorous cells stained more deeply than that of normal medullary cells (Fig. 2). The nuclei were not unlike those of normal cells of the adrenal medulla.

were not unusual for glands from animals of the age range.

Histologically the three large tumors consisted of cells with a slightly more basophilic cytoplasm and a slightly smaller nucleus and a less extensive cytoplasm than in the normal adrenal medulla (Figs. 6, 7, 8). As in the normal gland the cords were separated by vascular sinusoids supported by delicate connective tissue or reticulum. Mitotic figures were not numerous but one or several were not infrequently observed in one microscopic field. Fragments of or entire isolated adrenal cortical

TABLE 1

MICE OF THE BRS STOCK WITH ADRENAL MEDULLARY TUMORS (PHEOCHROMOCYTOMAS)

Mouse no.	Sex	Age at castration	Age at death	Location of tumor	Additional lesions
977	♂	2 mo.	609	Bilateral	Gastric tumor
989	♀	2 "	604	Unilateral	Gastric tumor, hepatoma
809	♂	1 "	598	Bilateral	Gastric tumor, hepatoma, hypertrophied mesenteric nodes
1206	♀	1 "	569	Unilateral	None

TABLE 2

ROENTGEN RAYED FEMALE MICE WITH UNILATERAL ADRENAL MEDULLARY TUMORS

No. of animal	Stain or group	Treatment	Wt. of uterus mgm.	Age x-ray started	Age at death (days)	Size of tumor (mm.)	Other tumors
12	AC_3*		22.6	32	590	8.6×7.1	Granulosa cell
25	C_{57}	Stilbestrol** pellet	26.9	33	596	5.0×4.2	Lung adenoma
40	A	Testosterone propionate	52.8	34	616	5.5×4.2	
58	A	Testosterone propionate	35.3	28	618	Microscopic	Heptoma
128	C_3H	Stilbestrol pellet	163.0	31	583	Microscopic	

*AC_3—($C_3H \times A♂$).

**Stilbestrol pellets—consisted of 1 part stilbestrol and 3 parts cholesterol. Testosterone propionate—injected subcutaneously, 1.25 mgm. in 0.05 cc. of sesame oil once weekly.

Hemorrhagic areas were scattered throughout the medullae. The sinusoidal network usually present at the boundary between the cortex and the medulla was lacking; the tumorous medullary cells encroached upon it and projected into the cortex. The smaller adrenal lesions in the mice of the BRS stock did not encroach upon the cortices of their respective glands so extensively. Histologically they were not unlike the larger tumors (Figs. 3 and 4).

Three of the x-rayed mice had large adrenal medullary tumors measuring 5 mm. or more in greatest diameter (Table 2). At the time the necropsies were performed it was thought that the tumors were of adrenal cortical tissue although their gross appearance was quite different than that of the tumors of this origin that had been observed in mice of other experiments (Fig. 5). They had a smooth, shiny surface and a translucent, pale, reddish color. The opposite adrenal glands

cells, were present in one of the tumors. Adrenal cortex could be identified only around a portion of the circumference of the tumors.

The two smaller tumors were histologically similar to the larger lesions although the cells were slightly smaller. They might not have been detected had not the larger tumors been studied, and had not mitotic figures been prevalent. The tumors replaced part of the normal medulla and cortex without enlarging the gland (Figs. 9 and 10).

The non-tumorous adrenal glands of the 76 mice in which medullary tumors were not observed showed no hyperchromatic nodules of medullary tissue and although several sections from each gland, or in many instances serial sections, were examined mitotic figures were not observed.

DISCUSSION

Whether castration or roentgen irradiation is related to the formation of the adrenal medullary

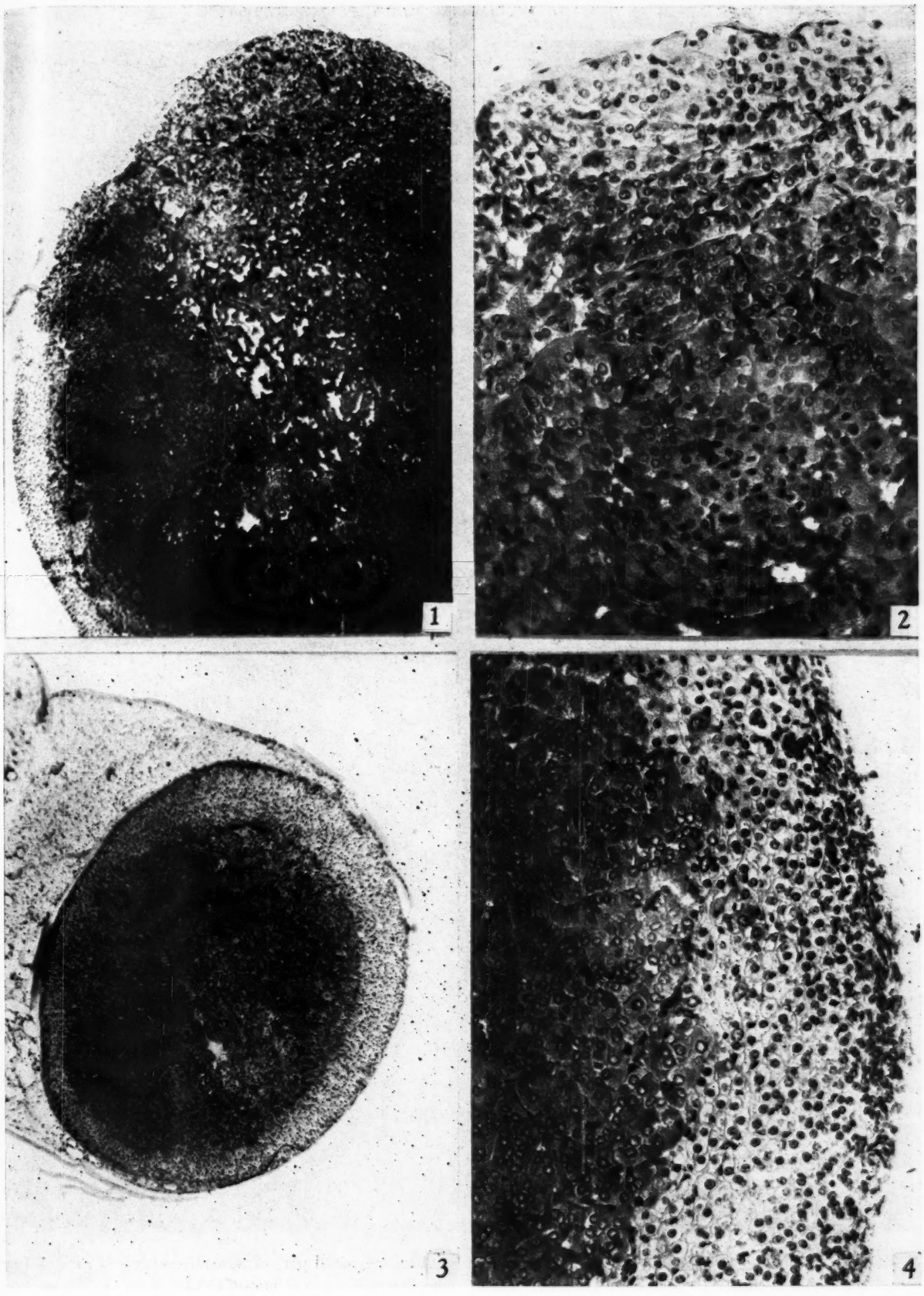
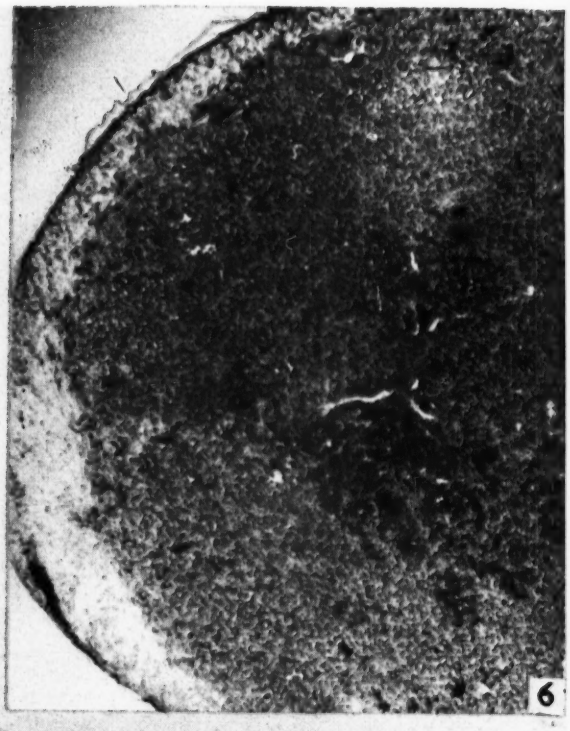


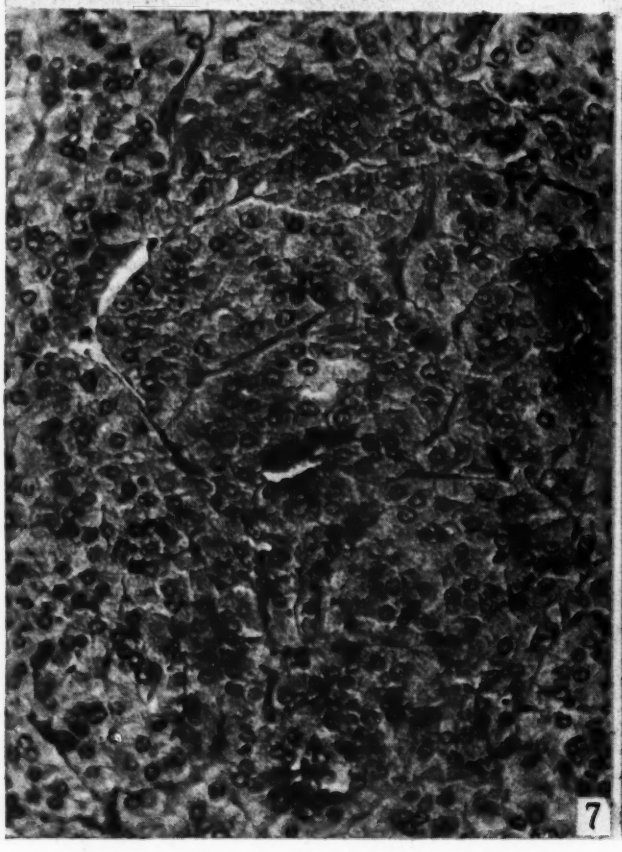
FIG. 1.—Cross section of the adrenal tumor of mouse 1206 (Table 1). A small amount of cortex surrounds the large medullary tumors on the lower left. 32 mm. objective and 10× ocular.

FIG. 2.—Section of pheochromocytoma shown above. ×195.

FIG. 3.—Cross section of small adrenal medullary lesion of mouse 977. 32 mm. objective and 10× ocular.

FIG. 4.—Section of adrenal cortex and adjacent abnormal medulla of mouse 809 (Table 1). ×195.





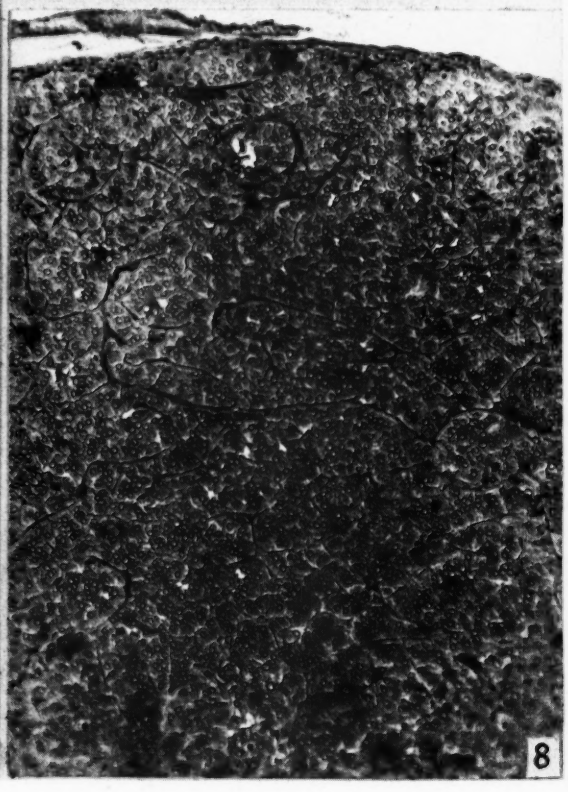


FIG. 5.—Photograph of urogenital organs and pheochromocytoma tumor of mouse 12 (Table 2). The large tumor is above the small left kidney. The right ovary was also tumorous. *Ut*-uterus. *B*-bladder. *OV*-ovarian tumor. *K*-kidney.

FIG. 6.—Small part of adrenal cortex and area of the pheochromocytoma of mouse 40 (Table 2).

FIG. 7.—Area of pheochromocytoma shown in Fig. 5 above. ×195.

FIG. 8.—Area of the pheochromocytoma from mouse 25 (Table 2). ×83.

tumors is certainly not proven. Many times the number of old animals, not treated in the same manner, were removed during the time the above experiments were in progress and no large pheochromocytomas were detected. Furthermore, the adrenal glands of many of the animals on unrelated experiments were studied histologically and medullary tumors of microscopic size were not observed. In fact, other than in these tumors, the mals so treated have acquired adrenal cortical tumors similar to those occurring in animals of some strains subsequent to castration (2) but medullary tumors have not been reported. Adrenal cortical tumors were not observed among the irradiated mice studied in these experiments. Most of the mice had been treated with gonadal hormones which might prevent the appearance of cortical tumors and were from strains in which large ad-

FIG. 9.—A small nodule observed in the adrenal medullary cells from mouse 128 (Table 2). Normal adrenal medullary tissue is shown below the small nodule, cortex above. ×83.

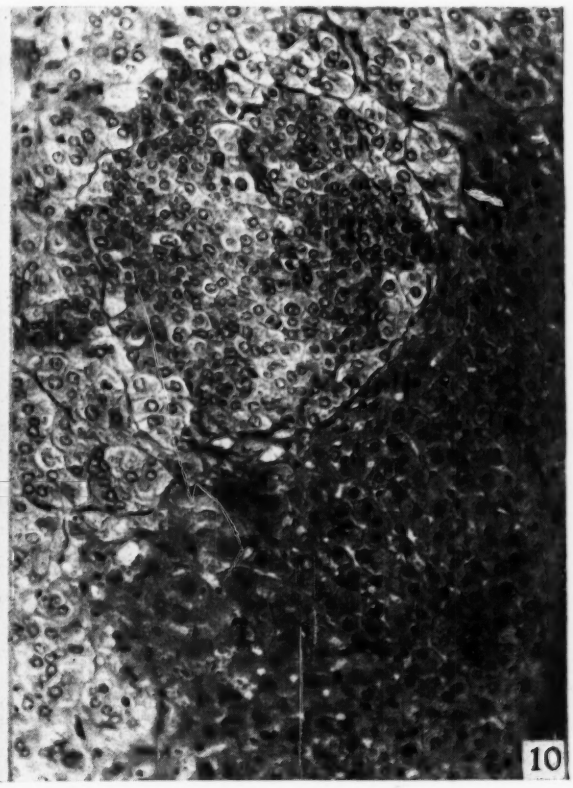


FIG. 10.—A small nodule of abnormal adrenal medullary cells from mouse 58 (Table 2). Two mitotic figures are shown. ×165.

writers have not observed mitotic figures in the adrenal medullae of adult mice. The lack of adrenal medullary tumors in groups of non-irradiated or non-castrated mice maintained in the same laboratory under presumably similar conditions, makes it seem improbable that other environmental conditions were responsible.

The tumors appeared in mice of 4 different strains and one hybrid mouse. The distribution among mice of diverse genetic origins makes it seem improbable that the mice have a genetic predisposition for such tumors.

Few adrenal medullary tumors in mice have been reported. They are presumably quite rare. Many mice have been irradiated and observed during their subsequent span of life. Occasionally adrenal cortical tumors rarely appear subsequent to castration. The one mouse that had received a pellet of stilbestrol-cholesterol had an atrophic uterus and no pellet was detected in the subcutaneous tissues. The pellet had been either completely absorbed or extruded. Two of the mice received more than enough testosterone propionate to maintain the accessory genital organs of castrated male mice. Under such circumstances it is difficult to believe that castration or the castration effects of roentgen irradiation were responsible for the appearance of the adrenal medullary tumors.

The tumors were not fixed in solutions containing dichromate and attempts to obtain a chromaffin reaction after the tissues were sectioned and placed on the slides gave weak or no response. Al-

though such conditions are not optimal to reveal the possible presence of chromaffin substances they indicate that the hormone content of the tumors may not have been high. Attempts to determine the presence of high levels of vasoconstrictor principle in the circulation by studying the blood vessels removed around, or in, the other tissues or organs saved were not too satisfactory. The age of the mice and coincident hormone treatment made interpretation of the results more difficult. The myometrial blood vessels of most of the mice given testosterone propionate and without adrenal tumors, had thinner walls than did those of mice with the pheochromocytomas. Unfortunately tissues other than those responding to the hormone or other than the tumors, were not saved.

SUMMARY

1. Four large pheochromocytomas were found in the adrenal glands of mice of different strains or hybrid groups that had been either castrated or irradiated (750 roentgen units in 12 days).

2. Small adrenal medullary lesions, histologically not unlike the large tumors, were noted in 5 other castrated or irradiated mice. These lesions are thought to be small pheochromocytomas.

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Histopathologic Study of the Mode of Inhibition of Cellular Proliferation by Urethane: Effect of Urethane on Walker Rat Carcinoma 256*

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INTRODUCTION

Urethane (ethyl carbamate) has cured no human neoplastic diseases but by means of its inhibiting effect upon cellular proliferation it has temporarily arrested the course of chronic leucemias (1, 4, 12, 22, 23, 24, 26), metastatic carcinoma (8), prostatic carcinoma (13) and carcinoma of the skin (1). It has thus given new impetus to the search for anti-neoplastic chemical substances. This search would be facilitated if the mechanism of action of urethane were understood. Haddow and Sexton (10) first demonstrated its inhibitory effect on cellular proliferation in animals using the Walker rat carcinoma 256. Besides a marked inhibition of this tumor, they described a histologic change toward increased differentiation and an increase in the fibrous stroma. Subsequent studies have led to the conclusion that these effects are secondary to a direct effect of urethane on the cellular nucleus seemingly arresting its mitotic activity (9, 15, 23). None of these studies, while studying the effect of this drug upon the dividing nucleus, has considered the role that the severe systemic effects of urethane (1, 11, 19, 20) may have in preventing the growth of tumors. The failure of cellular proliferation to occur in sufficient amount to heal surgical wounds in the presence of urethane was ascribed recently to a general metabolic effect rather than local action of the drug at the wound site (19). In experiments to be reported elsewhere (21) urethane even in near lethal concentrations was unable to inhibit proliferation of the vaginal basal epithelial cells in the estrus cycle when rats were supplied parenterally with estrogen or folicular stimulating substance. These experiments appeared to indicate that the inhibitory effect of urethane was indirect and was aided by the attending debilitation. Therefore the following study of the mechanism of inhibition of tumors by urethane was made in an attempt to evaluate in this process the relative roles of a general metabolic effect and a direct local inhibition of mitosis.

* This study was aided in part by a grant from the Committee on Growth acting for the American Cancer Society.

MATERIALS AND METHODS

One hundred and sixty-five adult male albino rats, obtained from Sprague-Dawley Farms, were used in these experiments. Depending upon their nutritional status their weight ranged from 140 to 320 grams, averaging 250 grams. Animals of comparable weight and age were used in any given experiment. The stock animals were maintained on laboratory chow.[1] Except in the first experiment, synthetic diets were used during the experimental observation period because they facilitated dietary control and manipulations. The synthetic diet most commonly used was a high protein diet containing 22 per cent casein and 30 per cent carrots (28).[2] In order to evaluate the role of inadequate protein intake or utilization on the tumor, two special synthetic diets (28)[3] were used. Both were similar in calories, fat and vitamins but the protein content of one was reduced to less than one per cent nitrogen and of the other was composed entirely of casein. The latter high protein diet differed from the casein-carrot diet mainly in that it did not contain carrots and liver concentrate since these contain some protein and therefore could not be used in the casein-free, non protein diet. In order to force feed anorexic rats treated with urethane, a semiliquid diet (7)[4] was fed by stomach tube to both

[1] Purina Laboratory Chow.

[2] Casein-carrot diet, composition per 100 gm.: casein, 22 gm.; carrots, 30 gm.; ruffex, 5 gm.; lard, 4 gm.; cornstarch, 22 gm.; salt mixture, 4 gm.; liver concentrate, 1 gm.; brewers' yeast, 2 gm.; water, 10 ml.; riboflavin, 500 microgm.; pyridoxine hydrochloride, 200 microgm.; calcium pantothenate, 200 microgm.; choline chloride, 100 mg.; vitamin A, 200 USP uiits; and vitamin D, 29 USP units.

[3] Casein diet, composition per 100 gm.; casein, 22 gm.; ruffex, 5 gm.; lard, 4 gm.; corn starch, 50 gm.; salt mixture, 4 gm.; water, 15 ml.; thiamine, 540 microgm.; riboflavin, 830 microgm.; niacin, 1340 microgm.; pyridoxine, 600 microgm.; calcium pantothenate, 1275 microgm.; choline chloride, 180 mg.; vitamin A 400 USP units; and vitamin D, 58 USP units. The non protein diet was identical except that the casein was replaced with 22 gm. of corn starch.

[4] Semi-liquid tube feeding diet, composition per 100 gm.: Amigen (Mead Johnson), 14.1 gm.; dextrin, 19.95 gm.; Mazola oil, 46.5 ml.; ruffex, 8.3 gm.; salt mixture, 6.6 gm.; water, 4.5 ml.; and vitamins as in the casein-carrot diet.

untreated and treated rats in two experiments. Wherever the food intake fell during treatment with urethane the diets were pair-fed to the consumption of the treated animals. Dietary consumption and body weight of each animal were measured daily.

Because Walker carcinoma 256 had been used by Haddow and Sexton (10) in testing the effect of urethane originally it[5] was used in all of these experiments. It was transplanted in 0.2 ml. doses subcutaneously over the spine as an aseptic saline emulsion of finely crushed, 10 day old tumor tissue diluted to 3 times the original volume. Occasional infections in the tumor were eradicated by adding 1000 units of crystalline Penicillin G to 1.0 ml. of emulsion before injection. In over 1000 transplants by this method this tumor never failed to take and never regressed. By 4 days sufficient growth occurred to form a small palpable mass. Starting at this time the length, width, and height of each tumor was measured by means of a vernier caliper every other day for 10 days. The resulting rectangular volumes although larger than the actual volume of the tumor because of the ovoid shape of the nodule were accurate enough to allow comparison of the growth of the untreated and treated tumors throughout the observation period. The logarithms of these volumes when plotted against the logarithm of the day of growth resulted in straight lines with a slope almost constantly reproduced in the control groups in the various experiments. In order to obtain a relatively more accurate ratio of inhibition as a result of treatment, the animals were killed 14 days after transplanting the tumor and the tumor removed and weighed. These data were analyzed for statistical significance and only those experiments with a p value for the difference of the mean tumor weights of less than 0.04 accepted for study. In one experiment, pairs of animals in the untreated and treated groups were killed daily starting 5 days after the beginning of the experiment and the tumors removed and weighed.

The tumors were fixed in formalin, imbedded in paraffin, sectioned at 4 to 6 microns and stained with hematoxylin and eosin, silver (27), Giemsa, and occasionally a tri-chrome stain (25). Acetic-orcein stains of crush preparations of the various tumors were made in order to study details of mitotic division. These preparations were all studied by the usual histologic methods. In addition quantitative morphologic studies were made by the method of Chalkley (2) of the per cent of tumor cells in mitosis and of the various components of the tumor; tumor nuclei, tumor cytoplasm, blood vessels, fibrous tissue and reticulum. This method for obtaining data on the frequency of tissue elements in a histologic section is based on the random distribution of 4 points in the objective of the microscope falling in the field of the section being studied. There is a minimum number of "hits," depending on the number of different tissue elements being counted, which result in reproducible data. In these studies at least 200 hits were recorded in studying the frequency of the various tumor elements and at least 400 total hits for mitotic frequency per slide. The averages based on 2000 to 3000 hits are statistically reliable figures.

[5] Obtained from Dr. H. P. Rusch, McArdle Memorial Laboratory, University of Wisconsin.

Urethane (Merck) was administered in 20 per cent aqueous solution intraperitoneally in doses of 500 mg. per kg. in a single injection. Ten animals in the first experiment received 2 such injections 12 hours apart daily. In all subsequent experiments only one injection was given daily to the treated animals starting on the fourth day after inoculation with the tumor.

Colchicine was used to arrest mitosis in one experiment, 1.5 mg. per kg. being given intraperitoneally. The animals were killed 7.5 hours later and the tissues studied for frequency of mitosis.

EXPERIMENTS AND RESULTS

The Effect of Urethane and Nutritional Debilitation on the Growth of the Tumor.—In the first experiment the effect of urethane was studied on the growth of the tumor in rats fed dog chow. Thirty rats were used: 15 received no treatment; 10 received 1000 mg. of urethane per kg. daily; and 5 received half of this dose. Previous preliminary experiments had shown that 14 days after transplantation the tumor increased in size very slowly and irregularly and became chiefly hemorrhagic and necrotic. Observations of the growth of the tumors in this and subsequent experiments were, therefore, limited to the first 14 days. Treatment with urethane was not started until the tumor had grown for 4 days since before that time it usually had not attained its maximum growth rate and was too small for measurement. The average growth curves of this experiment are shown in Figure 1. Urethane at the doses used inhibited the growth of the tumor and decreased the slope of the lines representing growth rate. The ratio of inhibition for the smaller dose was 3.4 and for the 1000 mg. per kg. dose was 5.2. Although the latter dose was given daily in two equally divided portions 12 hours apart, 6 of the 10 rats died on the fourth day and only 3 ultimately survived the 10 days of treatment. The average size of these tumors at 14 days is higher than it would have been if all the animals had survived and, therefore, this point falls far off the curve. None of the animals treated with half of this dose died. In nontumor bearing rats, 1000 mg. of urethane per kg. daily in divided doses kills only occasional animals in this period. Since the inhibition afforded by the smaller dose seemed adequate for our purposes, rather than determine the limit of tolerance to this drug of rats bearing this tumor, this dose was chosen as the standard one for all subsequent experiments. The casual observation in this experiment that the treated animals lost weight markedly led to strict dietary controls and body weight measurements in the following experiments.

The treated animals in the first experiment resembled closely in appearance the chronically protein-depleted rat. Since it was possible that urethane interfered with protein utilization by the animal and/or the tumor, the second experiment was done in which 5 rats[6] were depleted of their proteins over a 3 months' period by feeding them

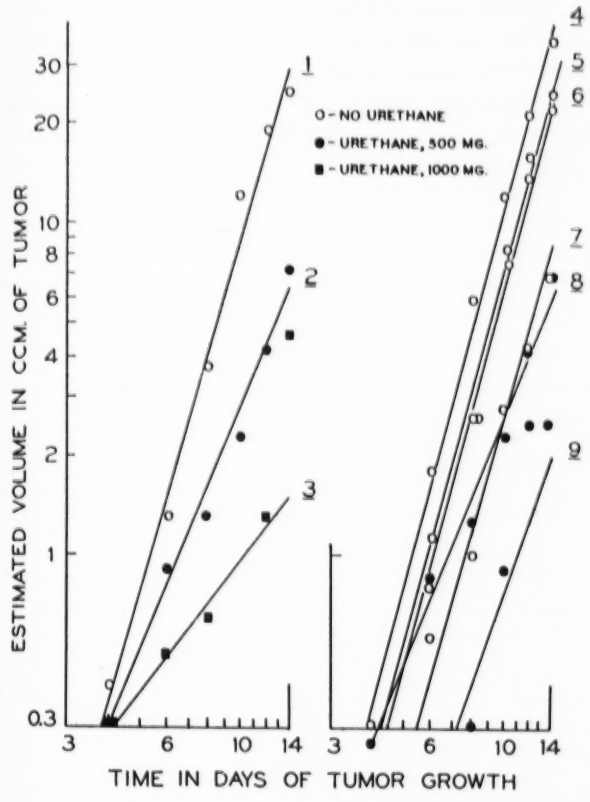


FIG. 1.—Maximum growth rates of Walker carcinoma 256 in rats fed various diets, with and without treatment with urethane, shown on logarithmic scales. The points represent the average estimated volumes of tumors on various days of growth in: *1*, normal rats fed laboratory chow; *2* and *3*, same treated with urethane at two levels; *4*, normal rats fed casein diet; *5*, normal rats fed by tube; *6*, normal rats restricted to the amount of diet eaten by the treated rats (*8*); *7*, protein-depleted rats receiving no dietary protein; *8*, rats fed the casein-carrot diet and treated with urethane; and *9* rats treated with urethane and fed by tube.

a diet extremely low in protein but isocaloric with the casein-carrot diet fed the 5 controls. No urethane was used in this experiment. After inoculation with the tumor, the protein-depleted group was placed on a protein-free diet which differed from the depletion diet by containing no liver concentrate or carrots but a larger amount of vitamins. The control group was given this diet with 22 grams of casein added per 100 grams. Both groups were offered 15 grams of diet per rat but the depleted animals consumed on the average only 9 grams daily. The resulting growth rates are depicted in Figure 1 and the data from this experiment are tabulated in Table 1. This severe depletion of bodily protein and deprivation of protein from the food caused a four-fold inhibition of the growth of the tumor. The latent period before the logarithmic growth rate was reached was prolonged. Once this period was passed the tumor in the depleted rats grew at the same rate as in the controls as evidenced by the similar slopes of the growth curves. The growth of the tumors in the rats eating the 22 per cent casein diet was comparable to that in the rats eating laboratory chow.

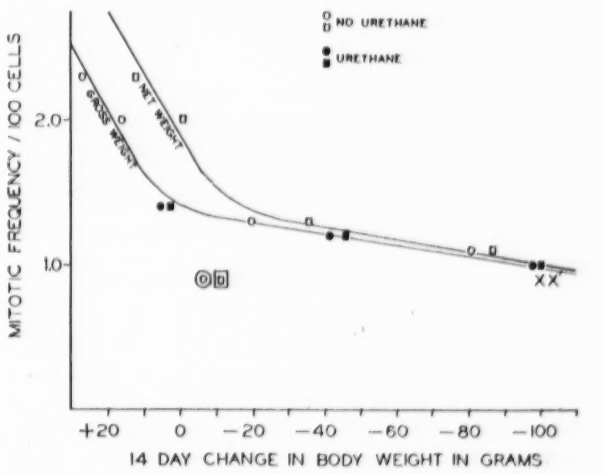


FIG. 2.—The relationship between the average mitotic frequency and the average weight changes of the animals resulting during 14 days of tumor growth with or without urethane therapy. The double lined points and their corrected locations at the X's are explained in the text.

These experiments concerning the protein nutrition of animals with tumors have been extended and will be communicated in a subsequent report.

In the third experiment, 4 days after the tumors were transplanted into 10 healthy rats, no food was given to them for the next 10 days. Five of these animals were treated with urethane. The results of this experiment are shown in Table 1. Starvation caused an average loss of 81 grams in body weight in the controls and 99 grams in the treated rats. Starvation alone retarded the growth of the tumor, with the controls of the previous experiment used for comparison, the ratio of inhibition being 3.2. Urethane and starvation on this basis caused almost a nineteen-fold inhibition.

In order to control the reduced food intake of the animals treated with urethane, the fourth type of experiment was performed. The untreated animals were fed the following day that amount of food consumed by the treated ones on the previous

[6] Obtained from Dr. P. R. Cannon, Department of Pathology, University of Chicago.

day. In various experiments of this type the average food consumption for the 10 day period varied considerably for no apparent reason. In Figure 1 and in Table 1 the data from the experiment with the best diet consumption by the treated animals are recorded. Restricting the controls to an average of 15.5 grams of diet per day did not retard the tumor but caused an average total weight loss of about 20 grams, the average net weight loss after subtracting the weight of the tumor was 36 grams. The treated animals had about a four-fold inhibition of the tumor, a total weight loss of 41.6 grams and a net weight loss of 46 grams. The slope of the growth rate curve of the treated tumors in this experiment corresponded to that of the treated tumors in the first experiment and not to that of the untreated tumors.

TABLE 1

THE RELATIONSHIP BETWEEN THE AVERAGES OF DIETARY INTAKE, TUMOR WEIGHT, AND BODY WEIGHT IN URETHANE TREATED AND UNTREATED RATS

Experiment number	Number of animals	Diet[1]	Daily diet intake average (grams)	Urethane 500 mg/kg/day	Av. weight on day 4	Av. net weight change (gms.)	Av. excised tumor wt. day 14	Av. gross wt. change	Av. mitotic frequency per 100 cells	Av. ratio inhibition	Av. ratio inhibition based on Exp. II
II	5	casein	14.8	0	234.8	− 1.3	17.29	+16.0	2.0		
	5	no protein	9.0	0	140.8[2]	− 10.7	4.31	− 6.4	0.9	4.0	4.0
III	4	none	0	0	288.7	− 86.5	5.47	−81.0	1.1		3.2
	5	none	0	+	300.0	−100.0	0.92	−99.1	1.0	5.9	18.8
IV	5	casein-carrot	15.5	0	302.6	− 36.1	16.52	−19.6	1.3		1.0
	5	" "	15.5	+	300.6	− 46.1	4.45	−41.6	1.2	3.7	3.9
V	8	tube fed	14.0	0	243.0	+ 12.9	14.03	+26.9	2.3		1.2
	8	"	14.0	+	237.0	+ 3.0	2.40	+ 5.4	1.4	5.8	7.2

[1] See footnote on diet composition in Materials and Methods.

[2] Depleted of protein reserves by three months' feeding of the casein-carrot type of diet without casein.

In the fifth type of experiment the animals of both the treated and untreated groups obtained nourishment only by stomach tube. The maximum amount of diet that could be given by this method in one day without injury to the rats was 14 grams, representing about 77 calories. The best *ad libitum* consumption of the casein-carrot diet by the treated rats represented 37 calories. The results of the best of these experiments with tube feeding are recorded in Table 1. The tumors of the untreated rats grew about as well as those in the other experiments but the treated group suffered an average inhibition of approximately 6 times. The resulting growth rates in this experiment are depicted in Figure 1. The controls gained on the average 27 grams while the treated group gained only 5.4 grams, average net weight gains being 12.9 and 3 grams respectively. No attempt was made in these experiments to account for this great discrepancy between the relatively large food consumption through forced feeding of the treated group and their small gain in body weight.

In Table 2 the data are recorded from the sixth growth experiment. The animals were treated in the usual way except that the average daily weight loss of the treated and untreated animals was kept as constant as possible by gradually restricting the consumption of ration by the untreated group down to 5 grams a day. The animals were killed in pairs from each group daily after the first day of treatment in order to obtain daily weights of the tumors, the relative inhibition of the tumors sequentially throughout the 10 day period and tissue for histologic study. This treatment did not significantly inhibit the growth of the tumors in the control group while a two-fold inhibition was finally obtained in the treated tumors. The largest ratios of inhibition were obtained between the fifth and the ninth days of treatment. This phenomenon was also seen between those days in most other experiments where the ratio of inhibition was calculated on the basis of volume instead of weight during the course of the study.

It was found in a small series of animals that when therapy with urethane was started on the day of transplantation of the tumor, the latent period of growth was prolonged 2 to 4 days and no greater inhibition of the tumor occurred than by the usual method of treatment. In another small series the treated animals were allowed to live after the 10 days of treatment was ended. None of these inhibited tumors regressed and all finally reached the large size of the untreated tumors and killed the animals.

Histologic Studies of the Effect of Urethane on the Growth of the Tumor.—Microscopic study of the 14 day old tumors treated with urethane revealed the histologic picture described by Haddow and Sexton (10) as the typical effect of this drug on Walker carcinoma 256. The tumor cells were increased in size in the treated animals; scattered cells were about double the usual size and had pleomorphic nuclei. While most of the tumor cells

had larger nuclei than in the control animals, their cytoplasm seemed to be increased to a greater extent (Figures 3 and 4). The treated tumors had an abundant fibrous stroma rich in reticulum while the normal tumors had little of these elements except near the peripheral pseudocapsule (Figures 7 and 8). These changes tended to decrease the number of tumor cells per unit area. Starvation accentuated these changes due to urethane (Figures 5 and 6) and seemed to augment the increase in fibrous stroma and reticulum (Figures 9 and 10).

tumors. At the time of the first sacrifice period, after 5 days of growth and one day of treatment, segments of previously existing reticulum and connective tissue with capillaries were scattered throughout and concentrated at the periphery of the nodules irrespective of treatment. Continuous reticular fibers in young connective tissue appeared first in both groups of tumors in noticeable and about equal amounts on the seventh day of growth of the tumors. In the urethane treated tumors at this time a fine reticular network was

TABLE 2

COMPARISON OF THE AVERAGE SEQUENTIAL CHANGES IN WALKER RAT TUMOR 256 IN CONTROL AND URETHANE TREATED RATS (EXPERIMENT VI)

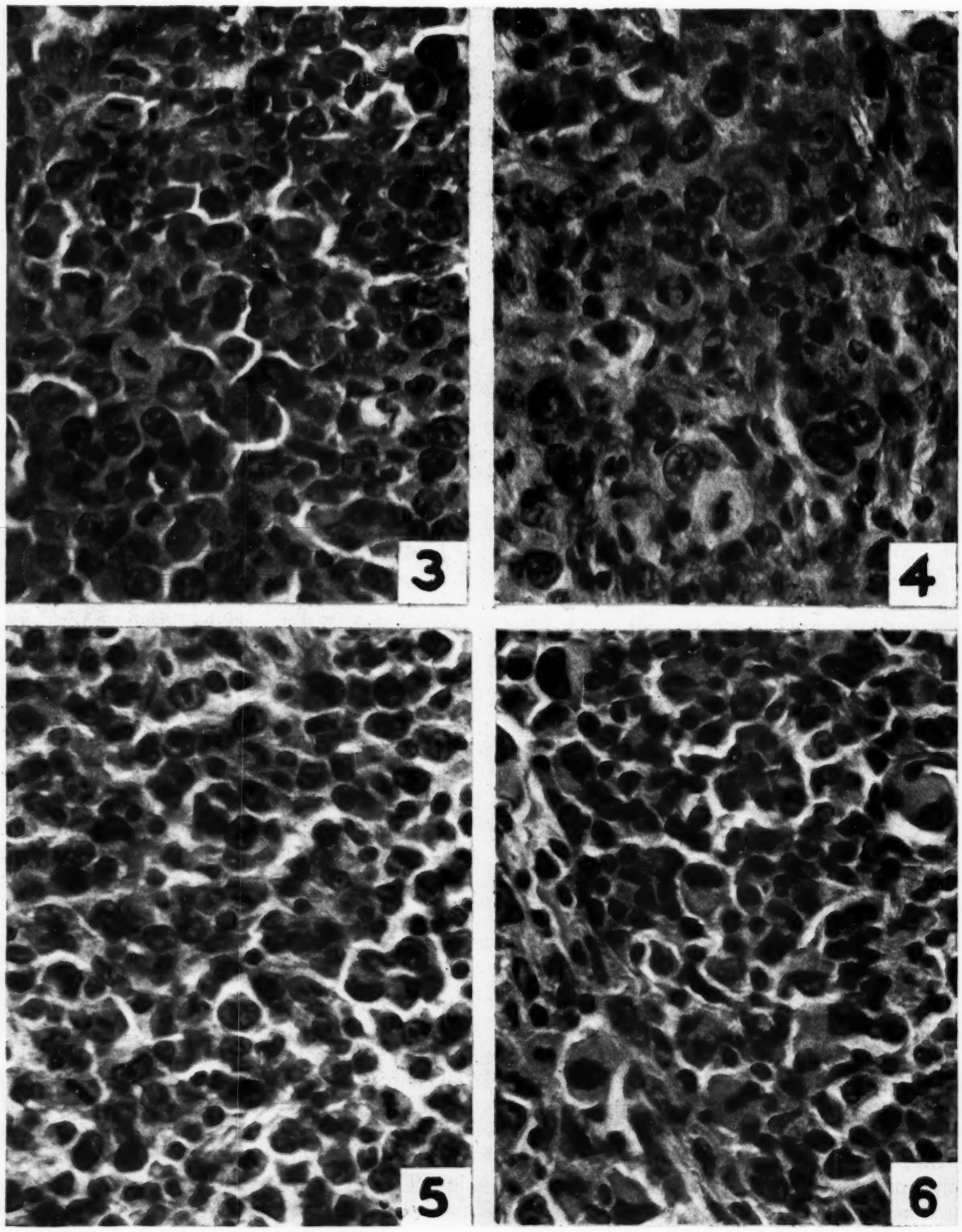
Day of treatment	Day of tumor	Number animals used	Diet intake daily* (gms.)	500 mg/kg urethane daily	Wt. on day 4 (gms.)	Net wt. change (gms.)	Excised tumor wt. (gms.)	Ratio of inhibition by urethane	Mitotic frequency /100 cells	Analysis of tissue components: % nuclei	% cytoplasm	% fibrous tissue	Cytoplasmic nuclear ratio
1	5	2	20	0	250	+ 1.3	0.72		1.8	18.0	77.0	1.3	4.3
		2	20	+	221	− 2.4	0.36	2.0	1.0	22.0	73.8	2.0	4.3
2	6	2	20	0	246	− 0.7	0.68		1.1	21.5	75.5	1.5	3.5
		2	16	+	252	+ 0.51	0.51	1.3	1.4	17.3	78.0	2.8	4.5
3	7	2	16	0	229	+ 1.0	1.55		1.7	21.0	76.0	1.0	3.6
		2	16	+	191	+ 0.8	0.69	2.2	1.1	20.8	74.8	4.0	3.6
4	8	2	12	0	237	− 8.4	2.36		2.5	20.8	75.0	1.5	3.6
		2	16	+	229	−11.1	1.13	2.1	2.3	23.0	70.3	5.3	3.1
5	9	2	8	0	264	− 8.5	5.99		1.9	19.3	77.0	2.5	4.0
		2	13	+	202	− 4.5	1.04	5.8	1.1	18.0	76.3	3.5	4.2
6	10	2	5	0	212	− 5.5	7.05		3.0	19.8	75.5	2.0	3.8
		2	9	+	269	−25.3	1.80	3.9	2.3	20.5	72.8	3.3	3.6
7	11	2	5	0	208	−14.0	6.90		2.4	25.5	70.0	3.0	2.7
		2	12	+	239	−12.2	2.18	3.2	1.7	11.0	80.5	3.0	7.3
8	12	2	5	0	229	−23.5	12.44		2.5	24.5	71.5	2.3	2.9
		2	17	+	232	−28.5	1.16	10.7	1.5	12.0	81.5	4.8	6.8
9	13	2	5	0	227	−27.5	10.96		2.0	25.0	73.0	1.0	2.9
		2	17	+	225	−25.0	4.02	2.7	2.1	13.5	77.5	5.0	5.7
10	14	3		0	233	−42.8	14.79		1.4	24.2	74.0	1.5	3.1
		3		+	233	−16.8	7.48	2.0	1.4	13.2	81.3	3.8	6.2

* Casein carrot diet.

Mitotic figures appeared to be as frequent in both treated and untreated tumors. No difference in number of capillaries was noted in either group of tumors except that there seemed to be greater dispersion of these vessels in the untreated tumors. The untreated tumors had more necrosis and hemorrhage than the treated tumors regardless of size. In general, however, the larger the tumor the greater was the amount of necrosis. The change in size of the tumor cells did not become manifest until the seventh day of treatment in the animals killed daily after administration of urethane was begun.

Study of the silver impregnated reticulum and the connective tissue stains of the tumors from the rats killed at intervals throughout the 10 days of treatment revealed that the amount of connective tissue varied with time and the growth rate of the

found diffusely throughout the nodules. This was not found in the untreated tumors until the next day. On the ninth day of growth, the reticulum in the central portions of the untreated nodules was becoming diluted by tumor cells while this did not occur in the treated tumors until the twelfth day, when these tumors increased relatively rapidly in size. The connective tissue and reticulum of the capsules of the tumors corresponded in amount up to the eleventh day when in the untreated animals peripheral extension of the growing tumor cells greatly dispersed these elements. These elements were similarly dispersed in the treated tumors by the thirteenth day but to a much lesser extent. In this experiment in which the urethane treated animals lost less weight than the restricted controls and the treated tumors grew better during the last several days than in other experiments, the



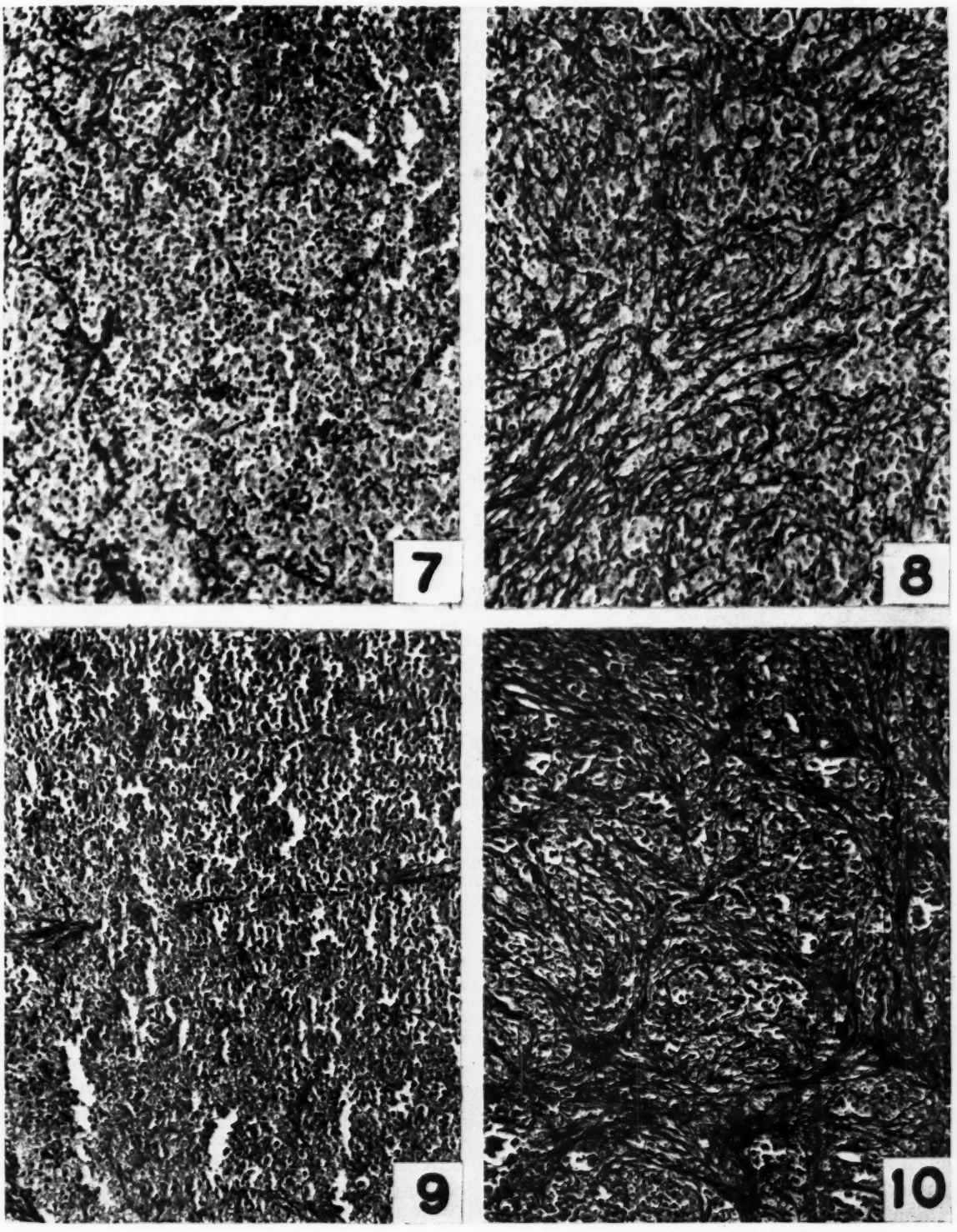
FIGS. 3 TO 6.—Photomicrographs of Walker carcinoma 256, 14 days old. Hematoxylin and eosin stains. ×570.

FIG. 3.—Untreated tumor from rat pair-fed the casein-carrot diet in experiment IV.

FIG. 4.—Tumor from rat treated with urethane in experiment IV.

FIG. 5.—Untreated tumor from rat starved in experiment III.

FIG. 6.—Tumor from rat starved and treated with urethane in experiment III.



FIGS. 7 TO 10.—Photomicrographs of the same tumors shown in Figs. 3 to 6, stained with Wilder's silver method for reticulum. ×125.

FIG. 7.—Same as Fig. 3.
FIG. 8.—Same as Fig. 4.
FIG. 9.—Same as Fig. 5.
FIG. 10.—Same as Fig. 6.

marked difference in the fibrous stroma and amount of reticulum in the two groups depicted in Figures 3 to 10 was not obtained.

These histologic impressions were confirmed by determining the frequency of tumor nuclei, tumor cytoplasm, fibrous tissue and capillaries in these sections by the quantitative morphologic method described above. These data are recorded in Table 2. The per cent of tumor nuclei decreased from 20.5 to 11.0 from the sixth to the seventh day evidently as the result of an increase in the amount of tumor cytoplasm and the bulk of the edematous stroma. Measurement of the tumor nuclei showed an acutal increase in their size. The incidence of mitotic divisions in the treated and untreated tumors from experiments II, III, IV, V (Table 1) and VI (Table 2) was determined by this method. The weight lost during the depletion period and is added to the weight lost during the period of tumor growth, this point then satisfies the curve at "X."

It did not seem likely that these small differences in mitotic frequency accounted fully for the differences in the size of the tumors treated with urethane. Therefore colchicine was used in an attempt to arrest the mitoses during a 7.5 hour period in order to determine whether the mitoses occurring in animals treated with urethane were delayed in completion and occurred at a slower rate. The results of this experiment are recorded in Table 3. Only a small number of rats was used and the arrest of mitosis by colchicine was not as marked as desired. A similar number of mitoses in the tumors and jejunal crypts were arrested by colchicine whether or not the animals were treated

TABLE 3

THE EFFECT OF COLCHICINE ON THE NUMBER OF MITOSES IN WALKER RAT TUMOR 256 AND THE JEJUNAL CRYPTS OF RATS AFTER TREATMENT WITH URETHANE, 500 MG., PER KG. PER DAY

		Tumor				Jejunum			
		Number mitoses per 400 cells days[1]		% mitoses days		Number mitoses per 10 crypts days		Number pycnotic cellular remnants per 10 crypts days	
Group	Animals used	8	11	8	11	8	11	8	11
Control	3	14	9	3.5	2.3	29	26	1	0
		12		3.0		32		1	
Urethane	4	14	10	3.5	2.5	39	30	7	0
		9	8	2.3	2.0	26	31	4	4
Colchicine	4	26	16	6.5	4.0	67	27	92	127
		27	14	6.8	3.5	71	39	101	108
Urethane and colchicine	4	26	18	6.5	4.5	89	44	49	56
		29	7	7.3	1.8	98	39	72	84

[1] Days after inoculation of the tumor.

control tumors were found by this method to have a consistently slightly higher frequency of mitosis than the treated tumors. The standard error in these determinations was ± 0.35, about half of what would be expected on the basis of the number of cells counted. This discrepancy probably is the result of the synchronous mitoses occurring in focal areas being picked out by the four pointers in the ocular of the microscope used in this method. Statistical analysis of these counts indicated that they were a reliable representation of the mitotic frequencies of the various tumors. Debilitation from undernutrition seemed to have as much influence upon mitotic activity as urethane as evidenced by the results in Table 1. Figure 2 depicts the relationship between the average mitotic frequency and the average weight change of the animals resulting from 14 days of tumor growth with or without urethane therapy. The circled point, that for the protein-depleted rats, is the only one that fails to fit the curve. If, however, the difference between the average weight of this group and that of its controls can be assumed to represent the with urethane. The apparent differences in these numbers in the jejunal crypts are resolved if the number of pycnotic remnants, considered to be cells injured in mitosis, are added to the number of actual mitoses. This dose of colchicine appeared to be less effective on the eleventh day than on the eighth day of tumor growth.

Among the mitoses of both untreated and treated Walker tumors a large proportion seemed abnormal. Some of such abnormal divisions result in cells that are incapable of further division, and except for their presence do not contribute to the continued increase in size of the tumor (17). A study of the numbers and types of abnormal mitoses in crush preparations of the untreated and treated tumors was made in order to determine whether these cells would be affected by treatment with urethane. The animals from which these preparations were made were tube fed in order to minimize the factor of nutritional debilitation. The results of this study are tabulated in detail in Table 4. The following were considered abnormal mitoses: polypoid prophase, large cells containing

a marked increase in number of scattered chromosomes some of which may have been fragmented chromosomes from metaphase; abnormal metaphase, clumped amorphous agglomerations of chromosomes at the equator of the spindle; sticking chromosomes, persistent attachment of chromosomes to one another after division and separation in anaphase; lagging anaphase, the lagging of one or more chromosomes behind the majority as they approach the asteroids; and bizarre figures, multiple spindles in a single cell. Among the mitoses of the untreated tumors about 50 per cent were considered abnormal; of the treated tumors about 76 per cent were abnormal. The greatest increases in numbers of abnormal mitoses in the presence of urethane were in the polypoid prophase and lagging anaphase, both of which result in daughter cells with abnormal numbers of chromosomes. Of interest is the close agreement of the average total number of prophases in the two groups when the numbers of normal and abnormal figures at this stage are totaled. The frequency of mitotic figures in these crush preparations, as determined by the method of Chalkley, was the same as that found in the paraffin sections of these tumors.

TABLE 4

ANALYSIS OF THE TYPES OF MITOTIC FIGURES IN CONTROL AND URETHANE TREATED WALKER RAT TUMORS
(STANDARD TUBE FEEDING DIET—EXPERIMENT V)

		Normal mitotic figures							Abnormal mitotic figures						
Animal no.	Mitoses studied	Prophase	Metaphase	Anaphase	Telophase	Degenerating cells	Total	%	Polypoid prophase	Abnormal metaphase	Sticking chromosomes	Lagging anaphase	Bizarre figures	Total	%
							Controls								
1	50	21	5	1	0	2	29	58	0	10	3	5	3	21	42
2	50	5	13	3	0	1	22	44	1	13	3	7	4	28	56
3	53	18	0	0	1	4	23	43	3	16	3	8	0	30	57
4	52	17	1	0	6	3	27	52	4	12	2	2	5	25	48
5	53	11	5	6	0	6	28	53	1	11	12	0	1	25	47
6	50	13	4	8	1	4	30	60	2	7	11	0	0	20	40
7	50	20	6	1	0	2	29	58	0	12	1	7	1	21	42
8	51	8	0	0	1	6	15	30	1	18	6	6	5	36	70
9	50	24	0	0	1	3	28	56	1	12	2	0	7	22	44
Average		15.3	3.7	2.1	1.2	3.2	25.5	50.0	1.40	12.3	4.8	3.9	2.9	25.3	50.0
							Urethane Treated								
1	50	2	0	0	0	3	5	10	3	11	9	19	3	45	90
2	54	2	9	0	2	1	14	26	8	14	5	11	2	40	74
3	50	10	2	0	2	1	15	30	10	11	9	5	0	35	70
4	51	12	1	1	1	3	18	35	5	22	4	1	1	33	65
5	50	8	0	0	1	0	9	18	12	19	3	5	2	41	82
6	50	12	1	2	0	1	16	32	8	14	4	6	2	34	68
7	50	4	3	1	3	1	12	24	6	21	6	1	4	38	76
8	50	0	5	0	2	2	9	18	4	17	10	10	0	41	82
Average		6.3	2.6	0.5	1.4	1.5	12.3	24.1	7.0	16.1	6.3	7.3	1.7	38.4	75.9

COMMENT

It would seem from these experiments that urethane in chemotherapeutic doses affects the proliferation of the malignant cells of Walker carcinoma 256 in the rat by both indirect and direct mechanisms. Urethane in tolerated doses, unlike colchicine, does not arrest or prevent mitotic division. The classification of urethane as an arrestor of mitoses is based on experiments which employed much more of this drug than is necessary for a chemotherapeutic effect. At near lethal doses urethane decreased mitotic activity and caused cellular degeneration in the crypts of Lieberkühn in the mouse (6), reduced reticulocytosis in rabbits (6), and inhibited all stages of mitosis in rat cornea for 8 to 12 hours (9). The cessation of mitosis in those experiments, however, does not necessarily represent a direct action of urethane, since an injection of estrogen restores mitotic activity in the vaginal epithelium of rats given such high doses of urethane daily (21). Phenylurethane, once thought to have an action exactly similar to colchicine (18), also, seems to have a mode of action that differs from that of colchicine in many respects (5, 10).

Urethane in doses at chemotherapeutic levels or above affects many organs and produces many severe systemic effects which could indirectly affect the proliferation of cells. Clinically patients receiving therapy with urethane lose weight out of proportion to their caloric deficit (1) and develop nausea, anorexia (14) and signs of systemic intoxication (13). A severe uncompensated alkalosis lasting 48 hours follows a single dose of 1000 mg. of urethane per kg. in rats and rabbits (11). Susceptibility to the lethal effects of x-radiation (11) and chloroform (20) is increased and the healing

of surgical wounds is indirectly prevented by this drug (19). In experiments reported here, rats treated with urethane and force-fed to prevent caloric deficit gained only one-sixth as much weight as the control animals. Control rats fed only that amount of diet consumed by the urethane treated rats lost only half as much weight as the latter animals. As shown in Table 1 and Figure 2 this systemic effect of urethane decreases mitotic frequency in the Walker tumor but no more than weight loss alone. The increase in size of the tumors is inhibited by urethane to a much greater extent than can be accounted for solely by this indirect effect of nutritional debilitation. The data from experiment VI (Table 2) seem to support this conclusion. After 14 days the severely restricted control animals had lost 42.8 grams in body weight and supported the growth of tumors averaging 15 grams in weight. The comparatively well fed animals treated with urethane lost in this time 16.8 grams in weight while developing tumors weighing only 7.5 grams. The only possible explanation for this phenomenon that was found in these experiments was the effect of urethane upon the number of abnormal mitoses in the tumor. As shown in Table 4, urethane caused a 50 per cent increase in the total number of abnormal mitoses. This effect appears most likely to be the result of some direct action of urethane within the nucleus similar to that of x-radiation (16), although no evidence was found for this assumption with the techniques employed in this study. In spite of the fact that no increase in numbers of atrophic and degenerating malignant cells was noticed in these tumors, it would seem that a possible explanation of the decreased growth of the treated tumors beyond that due to reduction in number of mitoses through debilitation, is the increased production of nonproliferating cells that results from the abnormal mitoses under the influence of urethane. A similar explanation has been advanced to account for some of the effect of x-radiation on the growth of malignant tissues (16).

From their study of the effect of urethane on the Walker carinoma Haddow and Sexton (10) gained the impression "that the profound modification in the histologic structure of the tumor by urethane . . . involves, in part at least, some attempted differentiation, and is not altogether to be ascribed to secondary changes involving the vessels and stroma only." The histologic studies reported here appear to show that the increase in stroma and reticulum is a reaction of the host secondary to the decreased proliferation of the malignant cells. No evidence of differentiation of the malignant cells toward a more epithelial character was found. The production, however, of polypoid enlarged cells and an increase in number of probably fatal mitotic abnormalities indicate that differentiation of malignant cells occurs during urethane therapy in the sense of a change from vegetative mitotic (proliferating) cells to fixed post-mitotic cells as defined by Cowdry (3).

No evidence was found to support the possible explanation that urethane affected the proliferation of malignant cells by restricting the growth of the nutrient blood vessels as it did in preventing wound healing when large doses of urethane were used (19).

SUMMARY

The effect of urethane on cellular proliferation in the Walker rat carcinoma 256 was studied in an attempt to determine the mode of action of this drug. By using easily manipulated synthetic diets it was possible to show that urethane inhibits cellular proliferation in part by nutritional debilitation of the animal. This indirect effect did not account for the total inhibition of the growth of the tumor. By means of histologic and cytologic techniques, urethane was found not to have an effect like colchicine but to cause an increase in the number of probably fatal abnormal mitoses in the malignant cells. The possible explanation was advanced that the inhibition of tumors by urethane beyond that due to altered metabolism was the result of the production of non-proliferating daughter cells by these mitotic abnormalities.

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Studies with the Polarograph on the Lipids of Epidermis during Normal and Rapid Growth*

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In a preliminary report evidence was presented to show that an alteration in the structure of a compound extractable from mouse epidermis with alcohol and ether occurs in the process of epidermal carcinogenesis in mice (3). In this paper observations lending further support to this structural change are presented.

MATERIALS AND METHODS

The essentials of the extraction procedures and the polarographic method were described previously (3), and any variations from those already given are in the proper place. Polarograms of the lipids were made in a mixture of unbuffered or buffered solution, dioxane, and tetrabutylammonium iodide (0.1 M). This quaternary ammonium salt was employed as supporting electrolyte to reach potentials in the region of −2.2 to −2.4 volts versus the saturated calomel electrode (S.C.E.) at which potentials some of the lipid material is reducible (3). All potentials in this report are in volts vs. the S.C.E., hence no further reference to the latter is given.

The epidermis of normal untreated mice and methylcholanthrene-treated mice was removed by blunt dissection at 50° C. on a thermostatically controlled hot plate by the procedure of Baumberger, Suntzeff and Cowdry (1). By this method practically pure epidermis is easily separated from the dermis as verified by microscopic study of both isolated portions of tissue. Even when the epidermis is removed at room temperature by scraping there is little, if any, contamination with dermis (7). The ideal nature of epidermis for the study of carcinogenesis, and the advantages of this tissue for chemical analysis have been discussed by Cowdry (5).

Methylcholanthrene-induced and three transplantable squamous cell carcinomas were used for this investigation. Some of the morphological characteristics of these tumors have appeared (3). In many instances transplantable tumors which were small, solid, and containing little, if any, necrosis were used for lipid extraction, and the preparation and advantages of these have been described (8). When large methylcholanthrene-induced or transplantable tumors were employed for analysis, the necrotic material was removed as thoroughly as possible with the aid of a magnifying lens. In all cases small pieces of every tumor were fixed and stained for histological control.

Polarography of the lipids was carried out in Heyrovsky vessels with a large anodic mercury surface, and the anode potential was determined for every sample. The anode potential was quite constant in all samples of similar composition with respect to dioxane, water, tetrabutylammonium iodide, and lipid. For example, the average anode potential of the lipids of normal and hyperplastic epidermis, and of the carcinomas in buffered and unbuffered solutions in 50 per cent dioxane solution for 80 determinations was −0.391 volts ± 4 mv.

The results for the lipid extractable materials in normal and methylcholanthrene-treated epidermis, which were polarographed in unbuffered solution, are shown in Table 1. The amount of lipid material is expressed as microamperes (diffusion current) per 100 mg. lipid, and the half-wave potential in volts. The diffusion current was determined on every sample and was proportional to the concentration of the lipid (Table 2). The half-wave potentials of the 2 waves were nearly the same when the dioxane concentration ranged from 37.5 to 62.5 per cent, and when the lipid concentration varied from approximately 2.5 to 6.0 mg. per ml. The potential values were quite constant in all cases since the pH was usually between 5.2 and 6.0. There was no significant change in the half-wave potentials in the lipid material from epidermis receiving many treatments with methylcholanthrene. Polarograms typical of the lipids of normal and hyperplastic mouse epidermis and of the carcinomas are shown in the preliminary report (3).

The amount of reducible material, as measured from the diffusion current of the second wave of the double wave, is expressed as microamperes per 100 mg. lipid. This average value for normal untreated epidermis was 25.0 μa per 100 mg. lipid, and for hyperplastic epidermis 52.4. The amount of reducible material in the latter tissue was twice that of the normal, and only 1 sample of hyperplastic epidermis contained less than any of the normals. Normal human epidermis[1] contained an average of 65.5 μa per 100 mg. lipid and is therefore in the same range as hyperplastic mouse epidermis. These two tissues have other similar chemical properties (4). The amount of reducible material extracted and the half-

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[1] The authors are indebted to Drs. J. Frerichs and Peter Thomas of the Staff of Barnard Free Skin and Cancer Hospital for the human material.

wave potentials were independent of the amount of alcohol used in the extracting medium.

When epidermis was extracted with ether alone or with a mixture containing 40 ml. of ether and 20 ml. of pure dioxane, the lipid thus obtained did not show the double wave when polarographed in the unbuffered solution. Apparently the reducible lipid of the epidermis is protein-bound and requires breaking down with ethyl alcohol prior to extraction. The need for splitting lipoprotein complexes prior to complete extraction of tissue lipids has been emphasized (2).

When methylcholanthrene-induced carcinomas and transplantable squamous cell carcinomas (Nos. 1, 2, 3) were extracted with a mixture containing 50 per cent alcohol and 50 per cent ether, as were normal and hyperplastic epidermis and the lipid polarographed, a great difference was noted from that of the latter tissues. At a lipid concentration of 5 to 6 mg. per ml. in 50 per cent dioxane solution, a wave was seldom present with a half-wave potential comparable with that of epidermis. On the other hand, if these same tumors were extracted with mixtures of 75 to 60 per cent ether, a single wave always appeared. Only a few induced carcinomas and 3 of 6 samples of tumors No. 2 gave this single wave when extracted with a mixture containing 50 per cent alcohol and 50 per cent ether.

The amount of reducible material extracted from induced and transplantable tumors, and the half-wave potentials are shown in Table 3. For the induced tumors only 4 of many extracted with a mixture containing 50 per cent alcohol and 50 per cent ether gave a single wave. The tumors extracted with different proportions of solvents showed varying amounts of reducible material, ranging from 4.7 to 20.3 μa per 100 mg. lipid. The half-wave potentials were from -1.64 to -1.70 volts (average -1.67). None of the transplantable tumors Nos. 1 and 3 gave the single wave when extracted with the 50 per cent alcohol: 50 per cent ether mixture, but when 60 to 75 per cent ether was employed as extractant, the amount of reducible material obtained was from 8.1 to 19.1 μa per 100 mg. lipid (Table 3). The half-wave potentials ranged from -1.62 to -1.70 volts (average -1.67). The behavior of this reducible material with respect to its extractability with different proportions of alcohol and ether indicates that the lipid is probably protein-bound.

BEHAVIOR OF THE REDUCIBLE MATERIALS OF EPIDERMIS IN BUFFERED SOLUTIONS

The lipid material of normal and hyperplastic epidermis was polarographed in mixtures of buffered solution, dioxane (50 per cent by volume) and 0.1M tetrabutylammonium iodide. Buffer solutions (0.1M) of acetic acid-sodium acetate and citric acid-sodium citrate were employed for the pH range of 4.40 to 7.3. The final concentration of the buffers in dioxane was 0.05M. The effect of dioxane upon the pH of the buffered solutions was considerable in that it raised the pH value by at least one unit. The dependence of the half-wave

TABLE 1

REDUCIBLE MATERIAL IN EPIDERMIS IN UNBUFFERED SOLUTION

Tissue epidermis	Microamperes per 100 mg. lipid	Half wave potentials* in volts						Alcohol in alcohol-ether mixture (per cent)
		First wave			Second wave			
		A	B	C	A	B	C	
	39.3		−1.49	−1.40		−1.74	−1.70	60
	22.7		−1.46	−1.49		−1.68	−1.65	25
Normal mouse	23.1		−1.53			−1.71		50
	20.5		−1.48	−1.48		−1.71	−1.65	50
	19.6	−1.50	−1.48		−1.65	−1.65		50
Hyperplastic mouse:								
3 paintings MC†	47.8		−1.51			−1.67		60
" "	60.9	−1.49	−1.45	−1.42	−1.70	−1.70	−1.63	50
" "	24.0	−1.52	−1.48		−1.66	−1.70		50
10 paintings MC	65.5		−1.49			−1.67		25
12 paintings MC	50.5	−1.47	−1.47		−1.67	−1.66		50
24 paintings MC	65.8	−1.48	−1.47	−1.42	−1.69	−1.68	−1.65	50
Normal human	72.0	−1.45	−1.42		−1.61	−1.61		50
	55.0	−1.50	−1.45	−1.44	−1.69	−1.72	−1.67	50

* A, in 62.5 per cent dioxane at ½ original concentration
B, in 50.0 per cent dioxane at ½ original concentration
C, in 37.5 per cent dioxane at ¼ concentration

† Methylcholanthrene

TABLE 2

RELATIONSHIP OF DIFFUSION CURRENT TO CONCENTRATION OF THE LIPIDS IN EPIDERMAL CARCINOGENESIS

Concentration of lipid (C) mg./ml.	Diffusion current (i_d) microamperes		$\frac{i_d}{C}$	
	Epidermis in unbuffered solution			
	First wave	Second wave*	First wave	Second wave*
4.0	0.49	1.06	0.123	0.265
6.0	0.87	1.47	0.145	0.245
8.0	1.14	2.10	0.142	0.263
	Carcinoma in buffered solution pH 4.32			
	Single wave		Single wave	
7.2	0.162		0.023	
11.0	0.270		0.025	
18.4	0.430		0.023	

* Sum of first and second waves.

potentials of the reducible material of normal and hyperplastic epidermis upon the pH of the solution is shown in Table 4. The pH values were those obtained with a glass electrode after electrolysis. For both normal epidermis (average of 4 samples) and hyperplastic epidermis (average of 2 samples), the dependence of the half-wave potential upon the pH was practically the same. The lipid material of both tissues had a half-wave potential of −1.50 volts for the first wave and −1.72 volts for the second wave in unbuffered solution (pH 5.2 to 6.0). Only a single wave appeared (at pH 4.43 to 4.52) with a half-wave potential of −1.40 volts, which increased to −1.50 volts at pH 5.73 to 5.80. The double wave appeared at pH 6.33 to 6.43; the half-wave potential of the first wave was −1.57 volts and that of the second an average of −1.68 volts, while hyperplastic epidermis had a single wave with a half-wave potential of −1.70 volts. The lipid of both tissues had only a single wave with a half-wave potential of about −1.72 volts at pH 7.1 to 7.3.

TABLE 3

REDUCIBLE MATERIAL IN CARCINOMAS IN UNBUFFERED SOLUTION

Number of samples	Microamperes per 100 mg. lipid	Alcohol in alcohol-ether mixture (Per cent)	Half-wave potentials (average) in volts
Methylcholanthrene-induced Tumors			
4	11.2	50	−1.67
2	6.4	25	−1.64
2	14.7	* 25	−1.70
2	4.7	* 40	−1.68
2	20.3	† 25	−1.66
Tumors 1, 2, and 3			
3	7.2	50	−1.62
2	18.0	40	−1.70
7	18.2	25	−1.66
2	8.1	* 25	−1.67
1	13.8	* 40	−1.68
7	19.1	† 25	−1.65
1	18.0	† 40	−1.69

* Tumors in alcohol overnight at 0°–4° C.

† Tumors in alcohol at room temperature for 1 to 2 hours, then overnight at 0°–4° C.

The wave height of the first wave was not appreciably altered by changes in pH nor by the buffers employed. The diffusion current of the reducible material as measured from the height of the second wave was determined per 100 mg. lipid and this data demonstrated that the height of both waves at pH 6.33 to 6.7, or of the single wave at pH 7.1 to 7.3 was about equal to that of both waves of the double wave in the unbuffered solution. Thus it was apparent that the half-wave potential of the first wave had coalesced with that of the second wave at pH 7.1 to 7.3 the two waves becoming additive. The half-wave potential of the second wave was also dependent upon the pH of the solution (Table 4). The single wave at pH 7.1 to 7.3 disappeared above pH 8.0 in boric acid-sodium hydroxide, sodium hydroxide-glycine, tetrabutylammonium iodide-tetrabutylammonium hydroxide buffers, and in Sorensen's buffer of pH 7.6.

When the lipid material of methylcholanthrene-induced or transplantable squamous cell carcinomas was polarographed in buffered solutions waves characteristic of epidermis did not appear. Only a wave with an average half-wave potential of −1.67 volts appeared in the unbuffered solution (Tables 3 and 4). Since this wave was absent in the buffered solutions, its presence in the unbuffered solution was due to some lipid substance acting as a buffer. The nature of this material which acts as a buffer is not yet known.

The lipid of the carcinomas did show a very small wave, the half-wave potential of which was pH dependent (Table 5) and the diffusion current was proportional to the concentration (Table 2). This wave had a half-wave potential of −1.34 volts at pH 4.43, −1.40 volts at pH 5.70, and −1.47 volts at pH 6.43, which was about 100 mv. less negative than that of the half-wave potentials of the first wave of normal and hyperplastic epidermis. This small wave was also found in the lipid substance of liver, skeletal muscle, and sarcoma 180. Other tissues have not yet been examined for its presence. Since the half-wave potential of this small wave was significantly less than that of the first wave of normal and hyperplastic epidermis, another compound is probably

TABLE 4

HALF-WAVE POTENTIALS OF THE REDUCIBLE MATERIAL OF EPIDERMIS IN BUFFERED SOLUTIONS

Material	Half-wave potential ($\pi_{\frac{1}{2}}$)	pH					
		Unbuffered	4.43–4.52	5.73–5.80	6.33–6.43	6.70	7.10–7.30
		Volts	Volts	Volts	Volts	Volts	Volts
Normal epidermis	First wave	−1.49	−1.40	−1.49	−1.57	−1.62	
	Second wave	−1.72	H*	H*	−1.65	−1.69	−1.71
Hyperplastic epidermis	First wave	−1.50	−1.40	−1.51	−1.57		
	Second wave	−1.72	H*	H*	−1.71	−1.70	−1.73
Carcinomas	First wave						
	Second wave	−1.67					

* H, hydrogen wave.

indicated. Moreover, the first wave characteristic of normal and hyperplastic epidermis was not found in the lipid extracted from many other mouse tissues when polarographed in the unbuffered solution. Apparently an acid medium is essential for the electrolysis of the substance indicated in Table 5 in spite of the fact that liver contains much more of this reducible material than does muscle and carcinomas. On the other hand an acid medium (buffers) is not essential for the electrolysis of the lipid compounds of epidermis. The wave of this substance in the carcinoma is present in the neighborhood of pH 7.0 as is the first wave of epidermis which is present at this pH. The wave of this substance in the carcinoma is present in the neighborhood of pH 7.0 as is the first wave of epidermis but with a half-wave potential 200 mv. more positive than that of epidermis. The necessity of using buffered solutions for polarography of organic compounds has been stressed (6).

TABLE 5

HALF-WAVE POTENTIAL OF THE REDUCIBLE MATERIAL OF MOUSE TISSUES IN BUFFERED SOLUTIONS

	pH			
	4.43	5.70	6.43	7.0—7.1
MATERIAL	Volts	Volts	Volts	Volts
Liver	−1.33	−1.41	−1.45	−1.53
Skeletal Muscle	−1.34	−1.42	−1.47	−1.51
Carcinoma	−1.34	−1.40	−1.47	−1.50
Sarcoma 37	−1.36*			

* Insufficient material for study at higher levels.

DISCUSSION

These studies on the polarographic behavior of the lipid material of normal and hyperplastic epidermis and squamous cell carcinomas in buffered solutions may necessitate some modification of the concept on a qualitative chemical change in carcinogenesis proposed in the preliminary report (3). The reducible materials present in the lipid of normal and hyperplastic epidermis which gave 2 waves are completely lacking or are chemically altered in the carcinomas whether induced or transplantable. The lipid material of the carcinomas only gave a single wave in unbuffered solution because some substance was acting as a buffer. This substance may or may not have any structural relation to the reducible lipid materials of normal and hyperplastic epidermis.

It is therefore apparent that the lipid of the carcinomas is qualitatively different from that of the tissue of origin, epidermis, in that a compound or compounds responsible for the 2 waves in the latter tissue is lacking or is chemically different from that found in the carcinomas. The chemistry of this qualitative chemical change and the locus of the reducible lipids in cellular particulates is now under investigation.

A compound having the same property of acting as a buffer in the unbuffered mixture of dioxane, water and tetrabutylammonium iodide was found in the lipid material extracted from mouse transplantable sarcomas 37[2] and 180,[2] lymphosarcoma,[2] undifferentiated lung carcinomas[2] and spontaneous mammary adenocarcinomas. In contrast, only the lipid, less firmly attached to protein, from mouse cardiac and skeletal muscle and spleen showed this same property although the adrenals, lymph nodes, brain, liver, ovaries, pancreas, and kidneys were examined. On the other hand, human squamous cell carcinomas behaved as did the mouse carcinomas in the unbuffered solution (Table 6) but insufficient material was available to ascertain whether the lipid compound was actually reducible or acted as a buffer. The half-wave potentials of mouse and human epidermal lipid were nearly the same in the unbuffered solution. This similarity suggests the possibility of a similar chemical composition with respect to the reducible materials. Furthermore, the process of skin carcinogenesis in both species appears to be similar from these polarographic studies.

The lipid material from skeletal muscle, liver, and squamous cell carcinomas was well as sarcoma 37 had a very small wave in buffered solutions with a half-wave potential which was pH dependent and which was significantly less negative than that of the first wave of normal and hyperplastic epidermis. The compound responsible for this wave was not present in normal and hyperplastic epidermis, and its role, as well as that of the other reducible substances in skin cancer formation,

TABLE 6

REDUCIBLE MATERIAL IN HUMAN SQUAMOUS CELL CARCINOMAS IN UNBUFFERED SOLUTION

Sample no.	Microamperes per 100 mg. lipid	Alcohol in alcohol-ether mixture (Per cent)	Half-wave potential in volts	Location of carcinoma
1	97.5	25	−1.69	Metastatic to liver
2	44.0	25	−1.73	"
3	95.0	25	−1.72	Metastatic to lymph nodes
4	52.0	25	−1.62	Left side of forehead
5	65.4	25	−1.67	Penis
6	98.0	25	−1.67	Metastatic to liver

[2] The authors are indebted to Dr. V. Downing, of the National Cancer Institute, Bethesda, Md. for the mice bearing sarcomas 37 and 180 and to Dr. W. U. Gardner of the Department of Anatomy, Yale University School of Medicine, New Haven, Conn. for undifferentiated lung carcinoma and for the lymphosarcoma.

must await fractionation of the complex lipid mixture obtained from normal cells and from cells undergoing carcinogenesis.

SUMMARY

Polarography of material extracted from tissues with alcohol and ether was studied in a mixture of unbuffered and buffered solution, dioxane, and tetrabutylammonium iodide.

Evidence is presented to show that an alteration in the structure of a compound or compounds extractable from mouse epidermis and carcinomas by lipid solvents occurs during the process of epidermal carcinogenesis in mice. The lipids of hyperplastic epidermis behaved polarographically as did those of normal epidermis.

The half-wave potentials of the reducible substances of epidermal lipid were shown to be dependent upon the pH of the solution employed for electrolysis. The half-wave potential of a compound characteristic of mouse skeletal muscle, liver, squamous cell carcinomas, and perhaps other organs was found to be pH dependent.

The reducible compounds of mouse and human epidermal lipid appear to be the same. Furthermore, the process of epidermal carcinogenesis in both species seems to be similar with respect to the polarographic behavior of the lipids.

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The Preparation and Properties of the Mitochondria and Submicroscopic Particles of Normal and Leukemic Mouse Organs*

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The mitochondria and submicroscopic particles which were employed in the serological studies described in the second paper of this series (2) were prepared by the method of Hogeboom, Schneider and Pallade (3). These authors have shown that when rat liver cytoplasm is fractionated by centrifugation in 0.88 M sucrose the isolated mitochondria have the same morphological characteristics and staining properties as do the mitochondria in the intact liver cell. A submicroscopic particle fraction rich in nucleic acid can also be obtained. In this laboratory the sucrose fractionation technique has been adapted to mouse spleen (4). In this paper the preparation and properties of similar fractions obtained from mouse liver and kidney, as well as spleen, will be described.

MATERIALS AND METHODS

Mice of the Akm strain (1), about three months of age, were used. The leukemia, 9417 (1), was transplanted by the intraperitoneal injection of leukemic tumor or spleen two weeks before the animals were killed. For the leukemic preparations about 50 mice were used; 100 mice of the same age were utilized for the normal preparations. The animals were killed by spinal fracture. The spleens, livers, and kidneys were removed immediately, chilled, cleaned, weighed, and homogenized in nine volumes of cold 0.88 M sucrose. All further operations were conducted in the cold. The nuclei and cell debris were removed by centrifuging four times at 600 × gravity for ten minutes in the horizontal yoke of an International centrifuge. The mitchondria were sedimented at 20,000 × gravity for 25 minutes in a Servall SS-1 angle centrifuge. They were washed twice with 0.88 M sucrose in the same way.

The mitochondria supernatant was recentrifuged to remove the last traces of mitochondria. It was then diluted to 0.44 M sucrose by the rapid addition of ice water, and centrifuged for four hours at 20,000 × gravity. The supernatant was optically clear. The sediment was suspended in 0.88 M sucrose, which was then diluted to 0.44 M and centrifuged as before. Both the mitochondria and submicroscopic particles were suspended in about 3 cc. of 0.88 M sucrose per gram of original tissue.

The suspensions were analyzed for nitrogen by Kjeldahl analysis and for pentose nucleic acid (PNA) and desoxypentose nucleic acid (DNA) by the orcinol and diphenylamine procedures as described by Schneider (5, 6).

RESULTS AND DISCUSSION

No DNA was found in any of these samples which were analyzed. This is in accord with previous experience in this laboratory; if precautions are taken to avoid breaking nuclei, and if the homogenate is centrifuged until the supernatant is free of nuclei on microscopic examination, no DNA is found in any cytoplasmic fraction.

The results of the PNA analyses are given in Table 1. The values on the spleen mitochondria are in agreement with others obtained in this laboratory, which have varied from 0.21 to 0.34 mg. PNA per mg. of nitrogen, with no apparent difference between the mitochondria from normal and leukemic spleen. The values for liver are similar to those found for normal rat liver (3). Whether the low PNA content found for the "mitochondria" from kidney is characteristic for the mitochondria of this organ is impossible to say on the basis of only 2 experiments. It may be noted, however, that the PNA content of the "large granules" of rat kidney (obtained by fractionation in saline) is only half that of the corresponding fraction of rat liver (7).

Whereas the isolated mitochondria seem to represent a clear-cut fraction of the cytoplasm, the

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submicroscopic particles isolated in these experiments include only a small fraction of the total submicroscopic nucleoprotein. In quantitative experiments on the PNA distribution in mouse spleen (4) it was found that with the centrifugal force employed here only 10 per cent of the PNA in the mitochondria supernatant of normal spleen, and 5 per cent of that in the leukemic mitochondria supernatant, remained in the sediment after one washing. Care was therefore taken to keep the speed and time of centrifugation the same throughout these experiments, so that about the same fraction of submicroscopic particles would always be obtained. The differences in the PNA content of the P fractions obtained from normal and leukemic spleen are probably due to the inclusion of varying amounts of smaller particles, which are much richer in PNA. In other preparations the PNA content has varied from 0.34 to 0.54 mg. per mg. of nitrogen for large particles from normal spleen and from 0.36 to 0.54 for large particles from leukemic spleen. The values for kidney are again low, in agreement with the low PNA content found for rat kidney cytoplasm (7).

TABLE 1

THE PNA CONTENT OF THE MITOCHONDRIA (M) AND SUBMICROSCOPIC PARTICLES (P) FROM NORMAL AND LEUKEMIC MOUSE ORGANS. EXPRESSED AS MG. PNA PER MG. NITROGEN.

ORGAN	NORMAL mg.PNA/mg.N	LEUKEMIC mg.PNA/mg.N
Spleen-MI	0.36	0.34
II	0.33	0.26
III	0.34	
IV	0.21	
Liver-M	0.16	0.16
Kidney-M	0.07	0.04
Spleen-P	0.30	0.62
Liver-P	0.41	0.57
Kidney-P	0.26	0.17

SUMMARY

1. The mitochondria of normal and leukemic mouse spleen and liver have been isolated by centrifugation in 0.88 M sucrose. A fraction of similar sedimentation properties, but lower nucleic acid content, has been isolated from mouse kidney.

2. A fraction consisting of the largest submicroscopic nucleoprotein particles has been prepared from normal and leukemic spleen, liver, and kidney, and its nucleic acid content determined.

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A Serological Study of Cytoplasmic Fractions from the Spleens of Normal and Leukemic Mice*

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One of the important problems in present day investigation of cancer concerns intrinsic differences which may be demonstrated between the cells of normal and malignant tissues.

A fruitful field for such studies has evolved through the use of differential centrifugation for the fractionation of cells, a procedure which yields isolated morphological components suitable for physiological, chemical, and serological examination (4), (5), (9). Claude (1946) (6), (7) has described in detail his latest method of fractionation of mammalian liver cells with the resultant segregation of (a) large granules (mitochondria) (b) submicroscopic particles (held to be microsomes), and (c) a supernatant component. Recently Hogeboom and his associates (11) have recommended the use of 0.88 M sucrose, rather than water or isotonic saline solution, for the isolation of intact cellular elements. Centrifugal fractionation of rat liver homogenates, prepared with the sucrose solution, produced mitochondria whose morphology and staining properties were preserved.

The antigenic properties of certain of these cytoplasmic fractions have been investigated (9), (10), (15), (2). These "heavy materials", obtained by various methods of treatment of a considerable number of tissues, have been found to be of intricate make-up and to exhibit varying degrees of species and organ specificity. Furth and Kabat (9) concluded that complement fixation tests did not distinguish between sedimented particles from chicken sarcoma and those from normal chicken spleen. However, the sera of rabbits given the sarcoma materials exhibited tumor-neutralizing properties which were in no way related to the complement fixing antibodies. Kidd (15) has reported that heavy materials from certain tumors may be differentiated from those obtained from normal tissues by use of complement fixation procedures.

In this laboratory we were particularly interested in the detection of antigenic differences between the intracellular components isolated from the spleens of normal and leukemic mice. Mitochondria and submicroscopic particle fractions, hereafter designated "M" and "P", were segregated according to the method of Hogeboom, Schneider, and Pallade from the spleens, livers, and kidneys of normal and leukemic mice of the Akm stock (11). A strain of lymphoid leukemia (9417) was employed (3). While all of these cytoplasmic materials were used for the production of antisera in rabbits, this first report emphasizes the results of serologic studies with the spleen antisera.

MATERIALS AND METHODS

Preparation of Antigens.—The methods of preparation and some of the chemical characteristics of the M and P fractions obtained from the spleens, livers, and kidneys of normal and leukemic mice are described in the first paper of this series. (1)

Upon completion of the segregation process we distributed these materials in amounts of 1 to 2 ml. in glass ampoules which were stored immediately at −20° C. As required for injection of animals, or use in serological procedures, the ampoules were removed from the deep freeze, the cell fractions thawed in a water-bath at 37° C. and then diluted according to their nitrogen content.

Preparation of Antisera.—Full grown rabbits (5 to 6 lbs.) were used for the production of antisera. All animals were bled prior to inoculation. Just before the time of use the M and P preparations were diluted with 0.88 M sucrose to contain approximately 0.1 to 0.2 mg. nitrogen per ml.

The rabbits (2 to 3 for each M and P preparation) received 6 injections of 1 ml. each at 2 day intervals by the intravenous route. The materials were apparently non-toxic since the rabbits did not exhibit any ill effects from such treatment and no animals were lost during the entire immunization procedure.

The rabbits were bled from the heart 7 to 9 days

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after the sixth dose. Preliminary tests of the individual sera demonstrated closely related titers: therefore, the sera from rabbits receiving identical fractions were pooled.

The sera were inactivated at 56° C. for 30 minutes and stored in the refrigerator at 4° C. Careful technique preserved the sterility of the sera and we did not use a preservative.

Complement Fixation Tests.—The complement (Lyovac) was titrated by the method described by Kent, Bukantz and Rein (14) for the determination of the 50 per cent hemolytic unit. Three such units in 0.2 ml. were employed in all tests. Ten per cent egg albumin was added to the buffered physiological saline solution used for dilution of the complement while all other reagents were diluted with the buffered saline solution. Each M and P antigen was diluted to contain 0.0055 mg. of nitrogen per ml.

The sera were diluted in twofold fashion and used in amounts of 0.2 ml. After addition of the complement and 0.2 ml. of antigen the mixtures were placed in the refrigerator for approximately 16 hours. After such incubation 0.4 ml. of sensitized sheep red cells (2 per cent suspension) were added to all tubes and readings made after a second incubation period of 30 minutes in a water-bath at 37° C. The highest dilution of serum giving a 2 + reaction was recorded as the titer.

Test for Forssman Antibody.—The agglutination procedure was used for routine tests. Equal volumes of diluted sera and 2 per cent suspensions of sheep red cells were combined and incubated at 37° C. for 30 minutes. Readings were made after overnight storage in the refrigerator.

Absorption Tests.—Since the material for study was limited, absorption tests were restricted to the M antisera and the corresponding antigens. The sera were absorbed 2 times with sheep cells, the supernates divided, diluted 1:2, and combined with equal volumes of the M fractions. An incubation period of 30 minutes in the water-bath at 37° C. was followed by overnight storage in the refrigerator, after which the preparations were centrifuged at 20,000 × gravity for 1 hour and the supernates used in tests. We used both the original sucrose suspensions and saline suspensions of mitochondria sedimented from the original preparations at 20,000 × gravity for absorption of the antibody.

RESULTS

General Properties of the Antigens and Antisera.—The M and P fractions are excellent antigens in that they stimulate the formation of antibodies of substantial titers. They are complex, rather than simple in nature since they exhibit organ, species, and fraction specificity. All fractions except those from the liver contain the Forssman antigen which is characteristic of mouse tissue preparations (9), (10). The heterophile antibody titers, as determined by agglutination tests, were consistently much lower than those demonstrated for the M and P fractions in complement fixation reactions. The sera obtained prior to immunization gave negative reactions with the M and P fractions, as well as with the red cells of sheep.

Agglutination procedures were used at times, and while positive reactions were obtained it is believed that complement fixation tests furnished our most reliable data. It is emphasized that we are well aware that we used a very sensitive complement fixation procedure. Many of our tests were repeated with less sensitive methods which reduced all titers but did not alter the pattern of overlapping reactions.

Our data are presented in 3 graphs, each of which depicts the comparative reactivity of antisera for either the M or P series of antigens. The serum titers were recorded numerically and then transferred to graph paper in order to express the relative titers for the various cytoplasmic components. The highest reading in any single titration of a serum was assigned the 100 per cent position and the others were given comparable ratings. For example, normal spleen M antiserum gave the following titers for the M series of antigens: normal spleen 1:1280, leukemic spleen 1:2560, normal liver 1:160, leukemic liver 1:320, normal kidney 1:320, leukemic kidney 1:160. The reading for the leukemic spleen M antigen was therefore recorded as 100 per cent with the others in proper relation. For the sake of simplicity the numerical titers are not included but it is repeated that they were all of substantial nature and ranged from 1:320 to 1:2560.

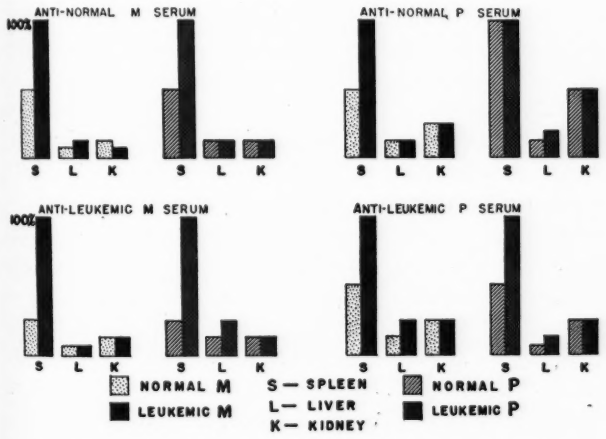
The Reactions of Spleen Antisera with the Spleen, Liver, and Kidney Fractions.—Graph I portrays the results of complement fixation tests using the spleen antisera and the cell fraction antigens isolated from the spleens, livers, and kidneys. The reactions with the liver and kidney fractions are included for contrast purposes.

It is evident that each of the spleen M and P antisera—whether produced in response to cell components from normal or leukemic animals gave reactions with the fractions segregated from normal and leukemic livers and kidneys, as well as those isolated from the normal and leukemic spleens. In no instance, however, did the titers with the liver and kidney fractions approach those obtained with the spleen components. As shown in Graph I the titers for the spleen fractions were from 2 to 16 times higher than those for the liver and kidney antigens. These differences thus confer upon the spleen antisera a marked degree of specificity.

It is significant that these spleen antisera did not give positive reactions in complement fixation tests employing blood from either normal or leu-

kemic mice as antigens. This supports our assumption that the cytoplasmic fractions were free of serum proteins. On the other hand the same sera gave positive reactions with spleen, liver, kidney, and lung extracts prepared by various methods, thus indicating the species relationship between the intracellular components of the spleen and those of other mouse tissues.

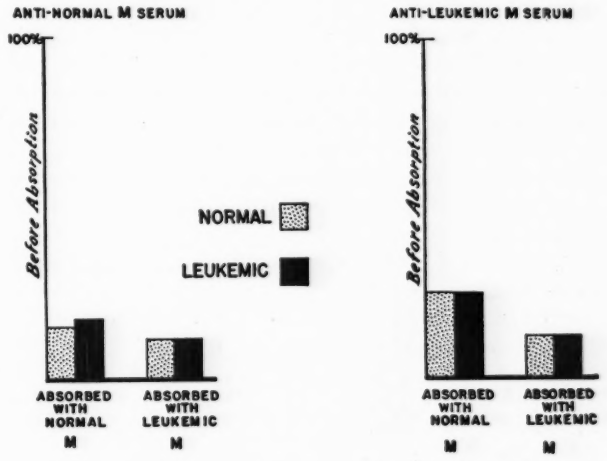
The most striking feature of the graph concerns the difference in the titers of the spleen antisera in favor of the leukemic antigens. All except the normal anti-P serum gave 2 to 4 fold higher titers with the leukemic than with the normal antigens. As stated earlier in the paper, our antigens were diluted at the time of test to give standard nitrogen values and every effort was made to secure uniform dilution. Had these differences represented isolated observations we would not have regarded them as significant. However, their consistency, and their persistence upon repetition of titrations, could not be ignored. These findings could suggest a quantitative difference in some antigenic component of the spleen cell fractions which was not measurable entirely by nitrogen evaluation. The same significant difference in reactivity between normal and leukemic antigens appears in certain of the liver fractions. On the other hand the cytoplasmic components from kidneys of these two groups of animals exhibited no such differences. Those quantitative differences interestingly parallel the probable leukemic infiltration of spleen,



GRAPH 1.—The relative titers of the spleen antisera for the M and P fractions segregated from the spleens, livers, and kidneys of normal and leukemic mice.

liver, and kidney. It would appear that there is an antigenic component present in both normal and leukemic spleens, but present in greater quantity in the leukemic than in the normal, which would explain the consistently higher titers with the leukemic antigen.

Graph II presents the results of absorption tests. Each serum was absorbed with the normal and leukemic M antigens after 2 preliminary absorptions with sheep cells. It is obvious that one exposure of the normal and leukemic sera to either the normal or leukemic antigen reduced, but did not remove completely the antibody for either antigen.



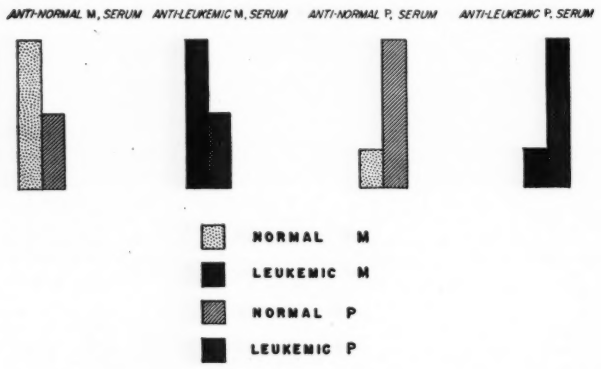
GRAPH II.—Showing the relative titers of the spleen anti-M-sera for the spleen M fractions after absorption with the normal and leukemic M fractions.

The data do not prove qualitative differences between the normal and leukemic antigens, but they point to the superior absorbing property of the leukemic fractions. The titer of the normal anti-M serum absorbed with its homologous antigen was reduced to 16 per cent of its original titer for the normal M component and to 17 per cent of its activity for the leukemic M fractions. When the same serum was absorbed with the leukemic M fraction the titers for both M antigens were reduced to 12 per cent. Likewise, absorption of the leukemic antiserum with the leukemic M antigen reduced the titers for both normal and leukemic fractions to 12 per cent. In contrast, absorption of the same serum with the normal M antigen reduced the titers for both antigens to only 25 per cent. The leukemic antigen thus removes more of the antibody from both the anti-normal and anti-leukemic sera than does the normal antigen.

Graph III depicts the relative titers of the anti-spleen sera for their homologous and heterologous intracellular fractions. There is indication that the M and P fractions of the normal and leukemic spleen cells vary in antigenic pattern. Because of the limited materials our data are restricted to those derived from titrations, but it is apparent that each of the antisera gives the highest reaction with its homologous cytoplasmic antigen. The normal anti-M serum exhibited a titer for the M frac-

tions which was 4 times that for the P fraction isolated from the same normal cells. In turn the normal anti-P serum reacted with the P fraction in a dilution 4 times that demonstrated for the M antigen. The same differences are repeated with the leukemic anti-sera. The M titer of the anti-M serum is 2 times that for the P fractions, while the P titer of the anti-P serum is 8 times that for the M antigen segregated from the leukemic spleen.

It is impossible at this time to draw conclusions regarding the character of the differences demonstrated between the cytoplasmic fractions of the individual cells. It should be repeated that each of these sera, whether from normal or leukemic mice, gave relatively higher titers with the leukemic than with the normal antigens.



GRAPH III.—The relative titers of the spleen antisera for the homologous and heterologous cytoplasmic fractions.

DISCUSSION

Centrifugal fractionation of the spleens, livers, and kidneys of normal and leukemic mice by the method of Hogeboom, Schneider, and Pallade (11), yields cytoplasmic components suitable for serological study. The fraction designated "M" was believed to consist largely of mitochondria on the basis of morphology and staining properties. The fraction designated "P" included submicroscopic particles which are assumed to vary in size and shape.

Mitochondria have been shown by Claude (7), Hogeboom (11), Kennedy and Lehninger (13) to be chemically complex and to contain many different enzymes. The P fractions are also chemically complex but as yet enzyme activity has not been demonstrated. We have found that both the M and P components are composite antigens in that they stimulate the formation of organ, species and fraction-specific antibodies. All fractions except those from the liver were found to possess in addition, the Forssman antigen. Antisera to the M and P fractions did not react in complement fixation tests with the sera of normal or leukemic mice, an indication that the cytoplasmic particles used as antigens were free of serum constituents. In general, these findings are in accord with those of Furth and Kabat (9), and Henle and his associates (10) in their studies of "heavy particles" derived from a number of tissues, but by different methods which were developed in their laboratories.

The M and P fractions used by us proved to be excellent antigens. Rabbits given 6 successive injections of 1 ml. amounts of suspensions estimated to contain 0.1 to 0.2 mg. nitrogen developed substantial titers of antibodies. None of the cell fractions proved toxic to rabbits. This is in contrast to Henle's experience (10) since he states that some of his organ particle suspensions produced death in a number of rabbits.

Especial attention was paid to the spleen fractions since we were particularly interested in detection of cytoplasmic differences between the cells of the spleens of normal and leukemic mice. When the spleen antisera were tested with the M and P fractions derived from spleens, livers, and kidneys a marked specificity was demonstrated, since the titers for the spleen fractions were from 2 to 16 times higher than those for the liver and kidney antigens. When it came to differentiation of fractions obtained from normal or leukemic mice we were not able to show qualitative differences between either the M or P fractions from the 2 groups of mice. In general the spleen antisera, whether produced in response to normal or leukemic cell fractions, gave higher titers for the leukemic than for the normal antigens. This superior combining power of leukemic fractions with antisera was again emphasized in absorption tests.

Interpretation of this consistent difference in favor of the leukemic antigens is not easy. Since all of the antigens used in complement fixation tests were diluted to contain a standard amount of nitrogen, it is believed that these observations may indicate a quantitative difference in some antigenic component of the spleen cell fractions which was not measurable entirely by nitrogen evaluation. Interestingly, the same differences, but much less marked, appeared in the liver fractions but not in the kidney components, thus paralleling the probable extent of leukemic infiltration of spleen, liver, and kidney in the diseased host.

Perhaps the most significant finding from our study had to do with the differences exhibited between the M and P fractions derived from either normal or leukemic cells. The anti-M sera gave substantially higher titers with the M antigens than with the P antigens. Likewise the anti-P sera gave higher titers with the P than with the M frac-

tions. These may represent structural as well as reactive differences but this must be established by further work. While the fractionation technic employed (1) was believed to yield materials with high degree of morphologic and chemical integrity, it is difficult to accept the premise that either M or P fractions could be prepared which would not carry some trace of the other cell constituent. However, the fact that the titers for the homologous antigens ranged from 2 to 8 times those for the heterologous fractions suggests substantial combining differences—perhaps a difference in antigenic pattern.

It is repeated here that all M and P antisera reacted in higher titers with the leukemic than with the normal antigens. These findings reflect the experiences of several investigators who have reported that antisera to normal tissues react with tumor preparations even more strongly than with the homologous antigen. Dmochowski (8) found that "immune sera against normal rat muscles contain antibodies which fix complement with rat tumor and rat embryo heated extracts even more strongly than with homologous antigen." He citse similar experiences of other workers. Kabat and Furth (12) found that precipitin tests did not "distinguish" between heavy materials from normal spleen and sarcoma. Nevertheless they show that the anti-normal serum gave slightly higher reactions with the tumor than with the normal spleen antigen and they state "the high speed deposit from spleen never reacted as strongly as that from tumor."

SUMMARY

The M and P fractions from the spleens, livers, and kidneys of normal and leukemic mice are of complex nature in that they possess organ, species and tissue fraction-specific antigens. All except the liver fractions possess in addition the Forssman antigen. Antisera to the spleen fractions show substantially greater reactivity for the spleen cytoplasmic antigens than for the liver and kidney fractions.

Antisera to all of the M and P fractions of both normal and leukemic spleens show greater reactivity for the leukemic than for the normal components.

Antisera to the M and P fractions of normal and leukemic spleens exhibit specificity of reaction with the homologous M or P fractions. The nature of these differences must be established by further work.

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The Effect of Walker Carcinoma 256 on the Total Lipid Content of Rats*

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The Walker carcinoma 256 growing in young rats may contain 20 to 40 per cent of the animals' total body nitrogen at death. A large proportion of the nitrogen in the neoplasm is contributed by the body's tissues since the amount of nitrogen stored during the period of tumor growth is much smaller than the nitrogen content of the carcinoma. In fact, the rat usually has a net negative nitrogen balance during the period of tumor growth under our experimental conditions. The total nitrogen deficit (the algebraic sum of the net nitrogen balance and the tumor nitrogen, the latter bearing a negative sign since the progressively growing tumor sequesters nitrogen) is considerable but constitutes a highly variable fraction of the total body nitrogen (2). It is apparent then that some other factor plays an important role in producing a fatal issue.

Earlier observations indicated also that depletion of the rat's body lipid progressed with growth of the Walker tumor. It now appears that the loss of fatty substances exceeds that caused by decrease in dietary intake, a universal finding among our tumor-bearing rats. This result suggests that growth of the Walker carcinoma 256 increases the caloric requirements of its host.

Sexually mature rats derived from the Wistar strain were housed in individual, suspended, basket-type cages. They were fed the following diet:

Casein, vitamin free	25
Sucrose	63
Crisco	8
Hubbell's inorganic salts	4
	100

The following mixture of vitamins was added to each 100 grams of diet:

Thiamin hydrochloride	0.001
Pyridoxine hydrochloride	0.001
Riboflavin	0.002
Inositol	0.005
Niacin	0.010
Calcium pantothenate	0.010
Choline chloride	0.100

The diet was mixed each week to preclude deterioration of riboflavin. Vitamins A and D were provided by feeding 0.25 ml. of cod liver oil U.S.P. once weekly. A separate weekly supplement of 40 micrograms of 2-methyl-3-phytyl-1,4-naphthoquinone was also fed.

The rats were divided into pairs of the same age, weight and sex. One rat of each pair was allowed to eat the ration *ad libitum*. The other rat was fed the amount of diet consumed by the experimental rat during the previous period. A fragment of Walker carcinoma 256 was introduced by trocar into the subcutaneous tissue of the inguinal region of each of the rats in which dietary intake was not restricted after the pairs had been observed for at least 1 week. The latent period of tumor growth was 7 to 10 days so that a minimum period of 2 weeks of normal growth was observed for each pair. The tumor-bearing rats were allowed to die of their neoplasms unless it appeared that ulceration of the tumor was imminent. The pair-fed controls were sacrificed at the end of the proper period.

The tumors were dissected from the carcasses and dissolved at room temperature in a 30 per cent solution of potassium hydroxide in 95 per cent ethyl alcohol. The gastrointestinal tracts were removed from the carcasses, opened from gastric cardia to anus, and cleaned. The carcasses were then dissolved in alcoholic solution of potassium hydroxide. The pair-fed control rats were treated in the same way.

The various solutions were diluted to standard volume and aliquots taken for determination of total lipids by a gravimetric method. Each solution was adjusted to approximately pH 3 with hy-

* This study was aided by a grant from the American Cancer Society, on recommendation of the Committee on Growth, National Research Council.

drochloric acid and extracted with a mixture of 6 parts of petroleum ether (BP 35° to 60° C.) and 1 part chloroform. The solvents were evaporated from the lipid extracts in a stream of clean air in a tared flask. The residue was dried to constant weight over anhydrous calcium chloride.

The pertinent data are listed in Table 1. Initial weight refers to the weight of each rat at the time the first evidence of growth of the tumor transplant was detected and the weight of the pair-fed control at the corresponding time. The final carcass weight indicates the weight of each tumor-bearing rat minus its tumor and the total weight of each pair-fed control. Each of the rats bearing Walker carcinoma 256 lost more weight from its carcass than did its control although the difference in total weights of each pair was much less striking. Three of the controls gained some weight though the other 23 lost weight, whereas the tumor-bearing rats gained in total weight during the period of tumor growth. A terminal antemortem period of precipitate weight loss characterizes rats bearing the Walker tumor. This period is usually associated with almost complete anorexia but some of the rats actually increase their food consumption during this stage although they do not gain weight. The three exceptional control animals were paired to tumor-bearing rats in which the latter phenomenon occurred so that the normal rats were able to increase their weight while the tumor-bearing rats lost.

TABLE 1

LIPID CONTENT OF RATS BEARING WALKER CARCINOMA 256 AND THEIR PAIR-FED CONTROLS

Tumor wt. in gms.	Sex	Initial weight in gms.	Final carcass weight in gms.	Total lipids Amt. in gms.	Total lipids Difference in per cent
22	F	176	126	4.4	−49
Control	F	175	153	8.6	
25	F	165	113	8.9	−56
Control	F	164	147	20.2	
25	M	205	176	5.8	−19
Control	M	206	186	7.2	
28	F	170	139	3.2	−53
Control	F	175	151	6.8	
29	M	225	198	9.0	−56
Control	M	231	219	20.3	
31	M	185	140	2.0	−51
Control	M	190	166	4.1	
32	M	239	157	2.7	−36
Control	M	238	185	4.2	
38	F	143	95	2.8	−61
Control	F	145	130	7.1	
40	M	187	143	2.6	−82
Control	M	191	181	14.4	
42	F	174	130	7.1	−32
Control	F	170	144	10.5	
47	M	230	180	8.4	−68
Control	M	235	215	25.9	
57	F	149	105	4.3	−71
Control	F	149	138	15.0	
58	F	172	106	2.3	−80
Control	F	185	191	11.3	
59	F	200	142	2.9	−86
Control	F	196	205	20.7	
61	M	167	115	2.0	−91
Control	M	176	180	21.6	
64	M	220	139	2.6	−66
Control	M	220	192	7.7	
65	M	208	164	4.3	−71
Control	M	210	198	15.0	
67	M	254	184	2.3	−87
Control	M	246	200	17.3	
71	M	163	123	3.0	−52
Control	M	157	168	6.3	
73	M	250	174	3.5	−73
Control	M	223	196	13.0	
75	F	185	127	2.2	−69
Control	F	195	165	7.2	
77	F	180	133	5.3	−69
Control	F	176	174	17.2	
86	F	194	116	3.8	−61
Control	F	192	168	9.8	
89	M	210	131	1.7	−85
Control	M	207	190	11.3	
105	M	248	160	2.1	−84
Control	M	256	242	13.2	

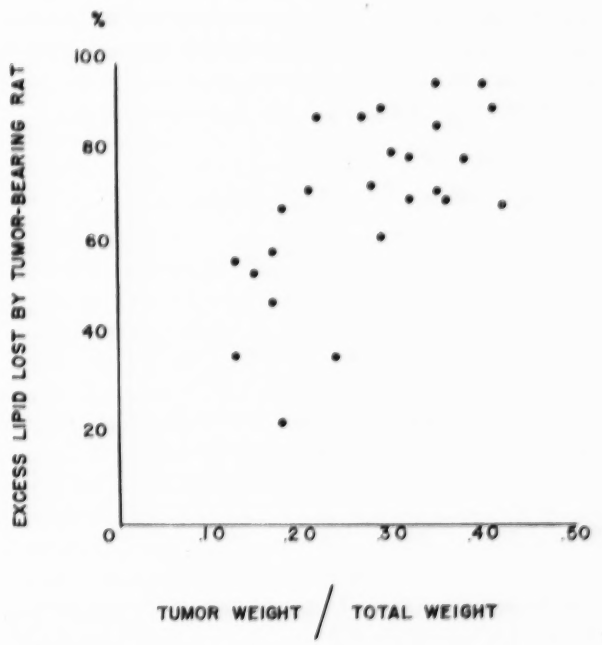

FIG. 1.—Excess lipid loss unrelated to dietary intake increases as the Walker tumor forms a larger proportion of the total weight.

Each of the rats in which Walker carcinoma 256 grew progressively contained less total lipid (carcass plus tumor) than did the pair-fed controls. This cannot be ascribed to decrease in dietary intake since the control and tumor-bearing rats ate precisely the same amounts of food. It is reasonable to assume that the rats of each pair contained the same amount of lipid at the onset of tumor growth since they had grown at the same rate for at least two weeks before the tumors appeared. This is borne out by observations of four pairs of normal rats in which the total lipid content of members of each pair varied less than 9 per cent.

Furthermore, numerous data on the lipid content of normal rats from the same colony eating the same diet indicate that the usual concentration is 9 per cent of body weight for females weighing 140 to 190 gms. and males of 150 to 250 gms. The excess lipid loss unrelated to dietary intake increases as the tumor forms a larger proportion of the total weight (Fig. 1). The precise relationship cannot be defined from our data.

It is probable that the depletion of total lipids affects chiefly fats though Aoki (1) found that the concentration of other lipid constituents was reduced in rats bearing transplanted hepatomas. Fat is an important reserve of food for the organism. It may provide energy when other sources fail. The greater loss of lipid in the tumor-bearing rat than in the pair-fed control suggests that growth of the Walker tumor imposes an increased energy requirement on its host under the conditions of our experiment.

SUMMARY

Sexually mature rats in which Walker carcinoma 256 grew progressively lost significantly more total lipid than did pair-fed controls of the same age, weight, and sex. Apparently growth of the Walker tumor increases the caloric requirements of its host.

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The Volume of Cell Nuclei in Amputation Neuromas and in Neurofibromas*

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The volume of nuclei and the changes in size that occur in various types of cells have been the object of numerous investigations. It is generally agreed that in tumors the volume of the nuclei is often greater than that which is found in the normal parent tissue. Also in skeletal muscle after injury or denervation, the nuclei are increased both in size and in number so that the total nuclear mass contained in a single fiber is increased.

The view has been advanced that in tumors this increased nuclear volume is due to an increase in chromosomic substance. In the case of damaged skeletal muscle the numerical increase of nuclei has been explained by Tower as due to stimulation from the degenerating nerve endings. This however does not account directly for the increase in nuclear volume which precedes proliferation. A toxic stimulus from degenerating nerve fibers has also been assumed to be the cause of the nuclear proliferation observed in nerve regeneration (Sunderland; Abercrombie and Johnson). In opposition to this view, one of us (R. A.) has advanced the opinion that "nucleosis" in damaged skeletal muscle and in damaged nerve is a release phenomenon due to a disturbance in pressure equilibrium, following the wasting of sarcoplasm, of axons or of myelin as the case may be. The "decompression" of nuclei may elicit osmotic changes and imbibition which would be directly responsible for the increase in nuclear volume. Such an enlargement of nuclei would naturally then lead to nuclear proliferation, since, according to the interpretation by Spencer, formulated by Levi, nuclei divide when they reach a certain volume ("critical phase"). It is well known to pathologists that Thiersch and later Ribbert assumed that loss of pressure or "tissue tension" was a cause of neoplastic growth, although they paid no particular attention to the reaction of the nuclei.

Opposed to the role of toxic products as the basis of changes of nuclear volume and of nuclear proliferation, and in favor of a mechanical interpretation, stand these facts: (a) ligation of severed nerves prevents the increase of nuclear volume; (b) tetanizing of skeletal muscle decreases the nuclear volume almost immediately; (c) curarizing of skeletal muscle with the ensuing muscle flaccidity is followed by increase in nuclear volume; (d) relaxation of tension by cutting of tendons leads to an increase in nuclear volume (Altschul, 2, 3).

In a previous publication enlargement of nuclear volume in experimental amputation neuromas has been reported (Altschul, 2). In 8 cases decrease of interstitial pressure was prevented by distal ligation of the stump. In seven of the eight cases no significant enlargement was observed. In hematoxylin-eosin preparations from four animals the ligated stumps showed a numerical increase of nuclei, which might be interpreted as neuroma-formation, but both in width and in length the masses were definitely smaller than in similar experiments without ligation. Silver preparations (Bodian) revealed in very few axons only an alteration in the form of growth clubs or Perroncito's spirals. Thus it may be concluded that ligation of the severed nerve prevents or hampers neuroma-formation. This finding is in agreement with the statement of Chenilleau that ligation of severed nerves prevents neuroma-formation and with the observation of Edds that coating the cut end of the nerve with synthetic resin achieves the same effect. White and Hamlin prevented amputation neuromas by covering the stumps with closely fitting tantalum caps.

There are other observations which support the view that the amputation neuroma owes its formation to the splitting of the connective tissue sheath with resulting "decompression" of the sheath cells. In 1917 Dustin described a kind of herniation of the nerve if the connective tissue sheath was slit laterally. Denny-Brown and Brenner reported a bulging of nerve fibers following a rupture of the perineurium, which would indicate that the nerve

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content is normally under a certain intraneurial pressure. If a nerve segment was resected but the connective tissue sheath preserved, no traumatic neuroma formed (Denny-Brown). This latter observation seems to repudiate the theory of toxic stimulation since under these circumstances the degenerating nerve fibers should produce sufficient toxic substances to elicit the development of a neuroma.

In our previous investigation we were able to present measurements from only one human amputation neuroma. It seemed worthwhile therefore to extend the examination to include more of these human amputation neuromas. At the same time it appeared promising to investigate the nuclear volume in neurofibromas (perineurial fibromas) and to compare it with that of normal nerves.

TABLE 1

AMPUTATION–NEUROMAS

	Normal segment					Neuroma					Difference
No.	Width*	St. error†	Length*	St. error†	Vol.‡	Width*	St. error†	Length*	St. error†	Vol.‡	in per cent
1	5.277	± .123	10.854	± .344	158.17	6.497	± .116	9.899	± .161	218.65	+ 38.24
2	5.224	± .133	11.134	± .296	159.00	5.719	± .123	10.315	± .216	176.55	+ 11.04
3	5.586	± .142	10.713	± .193	174.93	6.567	± .123	10.713	± .255	241.76	+ 38.20
4	5.551	± .107	11.332	± .261	182.73	6.965	± .125	10.731	± .186	272.41	+ 49.08
5	5.215	± .145	10.686	± .247	152.08	6.762	± .175	11.685	± .224	279.60	+ 83.85
6	5.056	± .107	11.287	± .335	150.99	6.612	± .121	11.685	± .210	267.33	+ 77.05
7	4.932	± .096	11.66	± .225	148.42	5.966	± .124	11.91	± .299	221.83	+ 49.46
8	4.906	± .133	11.92	± .347	150.13	6.302	± .108	10.72	± .194	222.79	+ 48.4
9	4.605	± .108	11.296	± .287	125.35	6.046	± .121	11.933	± .286	228.26	+ 82.1
10	5.533	± .114	11.199	± .179	179.41	6.523	± .110	11.553	± .238	257.24	+ 43.38
11	4.411	± .118	11.641	± .398	118.53	6.506	± .163	12.295	± .290	272.34	+129.8
12	4.349	± .116	10.545	± .289	104.37	5.807	± .127	11.04	± .259	194.81	+ 86.65

NEUROFIBROMAS

	Normal segment					Tumor					
1	6.178	± .136	10.412	± .208	207.96	7.381	± .180	13.347	± .424	380.51	+ 82.97
2	4.508	± .160	9.776	± .309	103.96	6.267	± .076	10.342	± .281	212.55	+104.45
3	5.768	± .127	10.65	± .202	191.15	7.668	± .224	13.195	± .457	408.55	+130.0

* In microns † Standard error ‡ Volume in μ^3

MATERIALS AND METHODS

Amputation (traumatic) neuromas and neurofibromas were received from the Army Institute of Pathology, Washington, D.C. (courtesy of Brigadier General R. O. Dart and Dr. W. Haymaker); from the Canadian Department of Veterans Affairs (courtesy of Dr. T. H. Williams); from the Department of Surgery, Columbia University (courtesy of Dr. Margaret R. Murray); from the Department of Pathology, University of Manitoba (courtesy of Dr. D. W. Penner) and from the Banting Institute, University of Toronto (courtesy of Dr. E. A. Linell). A great number of the amputation neuromas met the requirement of our technique, namely the presence of normal nerve along with the neuroma in the same section so that the sheath cell nuclei of the growth could be compared directly with those of the adjacent nerve segment, which, although perhaps not completely normal, had not participated in the nuclear proliferation. However, in spite of the relatively large number of neurofibromas at our disposal (32), only three preparations contained longitudinal sections of segments of normal nerve and could be used for the comparative measurements.

The nuclear measurements were made on sections stained with hematoxylin-eosin or with iron hematoxylin without counterstain.

The nuclei were chosen without regard as to whether they were neurilemma (Schwann cell) nuclei or nuclei of endoneurial fibroblasts. Length and width of the widest nuclei were determined by using a Zeiss ocular micrometer and an oil immersion objective. All the measurements were made by one of us (A.M.F.). The widest nuclei were selected for measurement because in these nuclei perspective shortening is of less influence in computing the volume. In previous controls, nuclei chosen at random had also been measured; the results so obtained agreed completely with those where only the widest nuclei had been selected. It was not considered necessary to attempt to measure the depth of the nuclei since it was assumed that the cross section is a circle. Although this is rarely completely true it has been established that in severed nerves (Abercrombie and Johnson; Denny-Brown) and in paralyzed muscle (Altschul, 3) the nuclei become rounder. If, therefore, we did oversimplify the calculation of the volume of the sheath cell nuclei, it is obvious that in amputation neuromas at least we minimized rather than exaggerated the differences between nuclei in the normal segment and those in the stump. Therefore the differences as shown in the last column of the table if they are inaccurate are smaller than they should be. It is probable that the same considerations apply to the neurofibromas.

From the length and the width the nuclear volume was calculated as a spheroid, $\frac{4}{3}\pi ab^2$. Thirty nuclei of the growth and thirty nuclei of the adjacent nerve segment were measured in cases 1 to 14, and sixty nuclei in case 15.

RESULTS AND COMMENTS

The results are shown in Table 1. The nuclear measurements on the amputation neuromas paral-

lel those obtained in animal experiments. As a matter of fact, they are even more consistent. The results thus obtained support the thesis that enlargement and proliferation of nuclei follow severance of nerve sheaths and are probably due to "decompression".

As to the neurofibromas, the small number of cases does not justify final conclusions. The fact that in our three cases of neurofibromas the sheath nuclei were much larger than those of the parent nerve, suggests that the factors at work here are similar to those in the amputation neuromas. This relation is emphasized if one considers the nature of the amputation neuroma, which, according to Ziegler is a "useless regenerative proliferation of a nerve stump, exceeding the physiological demand."

Tentatively the formation of neurofibromas may be considered as having been brought about by a hypothetical circumscribed weakness of the connective tissue sheaths by which intraneurial, but extranuclear, pressure would be lessened; this would allow for the enlargement and consequent division of cell nuclei. Such a hypothesis would be in line with the views of Thiersch and Ribbert. However, it should be pointed out that in the neurofibromas the degree of nuclear enlargement was more variable than was observed in the amputation neuromas.

SUMMARY

The enlargement of nuclei in damaged skeletal muscle and in severed tendons and nerves is reviewed. It is believed that a common factor in these different tissues is a decrease of extranuclear pressue leading to a secondary nuclear enlargement which in turn may result in nuclear division.

Sheath nuclei in twelve amputation neuromas and in three neurofibromas were measured. They were distinctly larger than the nuclei in the adjacent normal nerve segments. Some relations between amputation neuromas and neurofibromas are discussed. It is believed that in the former the nuclear increase is caused by a loss of pressure equilibrium, due to the severing of the connective tissue sheaths. It is possible that in neurofibromas some obscure weakness of the perineurium may be responsible for a similar loss of intraneurial but extranuclear pressure and thus for the ensuing nuclear enlargement and proliferation.

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Intratracheal Transplantation with the Brown-Pearce Carcinoma*

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Recently we became interested in studying the cellular components of bronchial secretions in cases of pulmonary neoplasms. For this study it became necessary to have an experimentally produced lung cancer in small laboratory animals. We required a tumor which grew in uniform fashion and which behaved biologically in similar manner in different animals of the same species. Furth (1) reported on the inoculability of mice lungs to transplanted tumor cells. The following report is a description of the technique of transplanting into and producing tumor growth in the lung of the rabbit.

MATERIAL AND METHODS

For this study we used Brown-Pearce carcinoma of the rabbit. This tumor was originally discovered and described by Brown and Pearce (2) in 1923. The tumor is a highly malignant epithelial neoplasm which was first successfully transplanted by Brown and Pearce into the testes. The tumor spreads by way of the lymphatics producing widespread visceral metastases. Histologically the tumor is an undifferentiated, anaplastic carcinoma.

Thirty-five rabbits were used for this study. Rabbits were chosen at random from stock supplies without regard to sex, color or weight. In general, large, adult, healthy rabbits free of respiratory infections, as far as could be ascertained, were chosen. Large rabbits were selected to facilitate laryngoscopy, the technique of which will be described below.

Tumor tissue used for the transplantation was prepared as follows: portions of healthy, actively growing tumor were removed from stock tumor rabbits, using aseptic surgical technique. Necrotic portions of tumor were discarded. The tumor tissue was minced with a curved scissors and further crushed in a tissue press. A small amount of isotonic saline was added to the crushed tumor tissue in sufficient quantity to produce a thick suspension. This thick suspension was used for transplantation.

For transplantation the rabbits were anesthetized by intravenous administration of nembutal. A solution containing 25 mg. of nembutal per cc. was administered intravenously until the animals were lightly anesthetized. About 1 cc. of this solution per kilogram of body weight was usually required. With the animals anesthetized a small, child's type, laryngoscope was introduced enabling direct visualization of the vocal cords. A size 8 French rubber catheter was introduced through the laryngoscope into the trachea and gently inserted to the bifurcation. Two cc. of the suspension of tumor tissue which had been previously prepared was then injected with gentle pressure from a Luer syringe through the catheter into the lumen of the trachea. The laryngoscope and catheter were then removed and the animal held in a vertical position to permit drainage by gravity of the injected tumor tissue into the lungs.

RESULTS

Successful tumor growth was obtained in 29 or 83 per cent of the 35 rabbits transplanted with tumor by this technique. It should be noted that the six rabbits in which there was no growth of tumor all comprised a group which were transplanted from the same batch of tumor tissue. The reason for the unsuccessful transplants cannot be determined, but it is quite likely that the tissue did not contain viable tumor cells.

Animals were sacrificed at varying intervals beginning at the 6th day after transplantation. At the 6th day tumor could not be identified grossly in the lungs. However, at this stage tumor could be readily identified microscopically (Figs. 1 and 2). On the 10th to 12th day following transplantation small nodules of tumor tissue could be identified grossly in the lungs and occasionally in the mucosa

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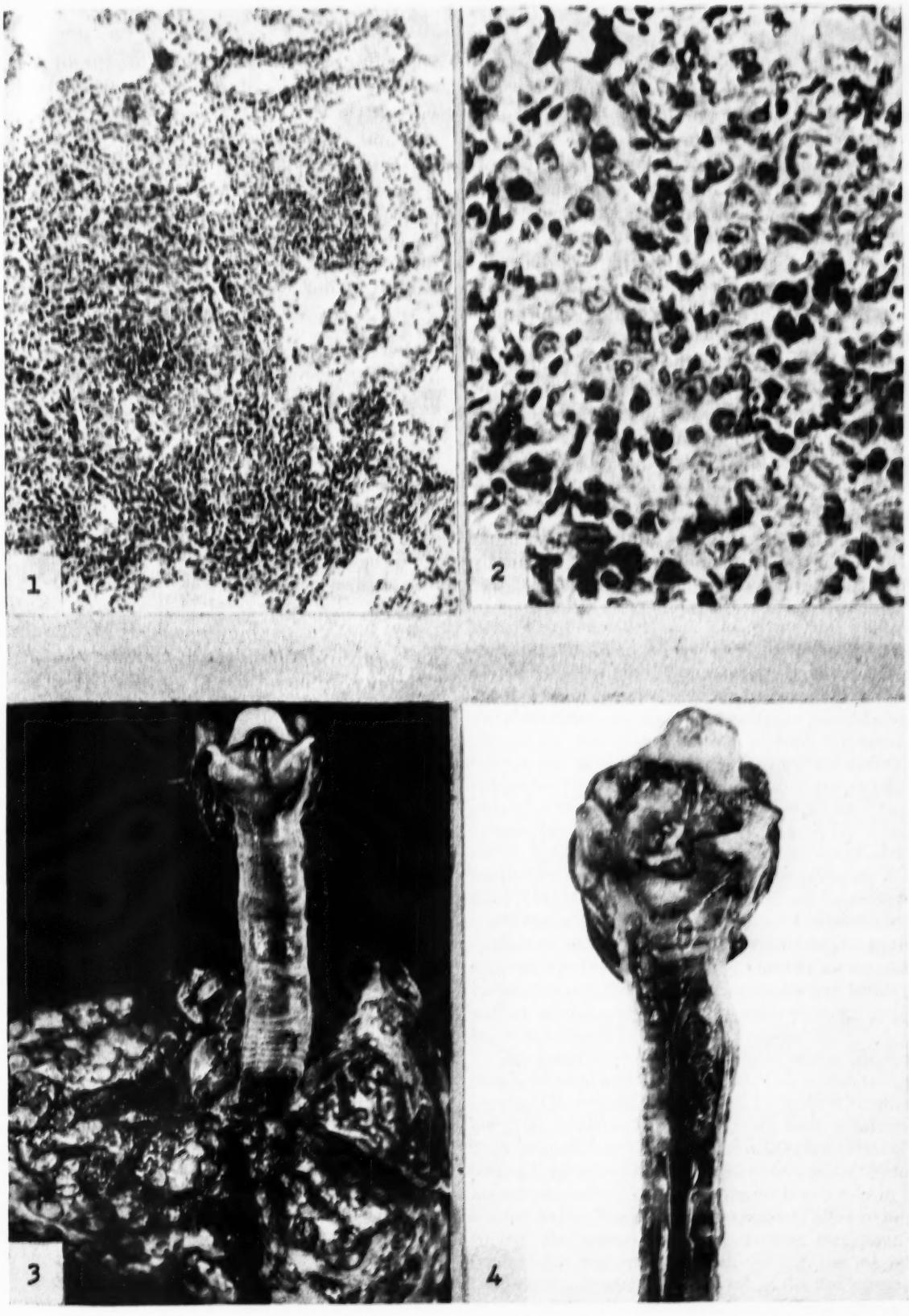

FIG. 1.—Brown-Pearce carcinoma nodule in lung, 6 days after transplantation when tumor was not visible grossly. (Hematoxylin and eosin preparation. ×100.)

FIG. 2.—Similar field as Fig. 1. (Hematoxylin and eosin preparation. ×400.)

FIG. 3.—Upper respiratory passages and lungs of rabbit that died 4 weeks after intratracheal transplantation with Brown-Pearce carcinoma. Note multiple tumor nodules throughout both lungs.

FIG. 4.—Larynx and trachea of rabbit that died 32 days after intratracheal transplantation with Brown-Pearce carcinoma. Note tumor nodules in wall of trachea.

of the trachea and bronchi. Since in the early stages tumor was found only in the lungs, it seems likely that the tumor growth within the bronchi and trachea was secondary to the "primary" tumor within the lung. Death usually occurred between the 21st and 38th day after transplantation. In animals which were permitted to die of tumor growth, both lungs were studded with myriads of tumor nodules measuring up to 1 cm. in diameter (Figs. 3 and 4). Death in these animals was usually due to asphyxia from occlusion of the stem bronchi by tumor masses and frequently superimposed pneumonia. Metastases to the liver were observed in a few cases. It should be pointed out that the absence of higher incidence of metastases was probably due to the fact that the animals died of the massive involvement of the lungs by the tumor growth before the neoplasm had had adequate opportunity to become disseminated.

Histologically the tumor nodules in the lungs were similar in their morphology to that originally described in testes and other organs. Necrosis of the tumor occurred early. The tumor was very cellular consisting of masses of epithelial cells with very little stroma. The cells varied markedly in size and shape as well as intensity of staining reaction and numerous atypical mitotic figures were seen. The cells varied from round or oval to polygonal. They had abundant, coarsely granular cytoplasm and pale, round or oval, vesicular nuclei containing one or more prominent nucleoli. In the advanced tumor areas of necrosis were common.

SUMMARY

A technique of transplanting tumors into the lungs of rabbits is described. Successful transplantations were accomplished in 29 of 35 rabbits used in this study.

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Arginase Activity and Nitrogen Content in Epidermal Carcinogenesis in Mice*

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This is one of a series of investigations the purpose of which is to characterize the nitrogen metabolism of mouse epidermis in various phases of growth and in carcinogenesis. It was reported previously that mouse epidermis has high concentrations of urea and preformed ammonia (1) and considerable arginase activity (2). The purpose of this communication is to report data on arginase activity in the epidermis of mice undergoing carcinogenesis as a result of treatment with methylcholanthrene in benzene and in control mice. These include animals treated with benzene alone or with a dilute solution of croton oil in benzene, a non-carcinogenic irritant. Finally correlative data on total nitrogen, trichloroacetic acid-soluble nitrogen (TCA-soluble N), urea, and preformed ammonia contents will be presented.

Reports in the literature give the general impression that the arginase activity of malignant tissues is higher than that of normal tissues, and this has led to the suggestion that the increased arginase activity may be associated with accelerated rates of protein synthesis and cell division (3, 4). Liver tumors, however, have much less arginase activity than the normal parent tissue (5). Probably the chief function of arginase in the liver is to form urea from arginine as part of the ornithine cycle. However, the function of arginase in the extrahepatic tissues is not known. The presence of large quantities of urea and preformed ammonia (1), and the detection of free arginine (6) and arginase activity (2) suggest the possibility that the ornithine cycle may also be operative in mouse epidermis. This is under investigation at the present time. It has been suggested that arginase may participate in the transfer of the amidine group of arginine to glycine to form guanidoacetic acid in the kidney (7). This enzyme may also be concerned with the metabolism of the arginine-rich histones. None of the roles suggested for arginase in the cellular economy has received suitable quantitative expression because most of the methods for estimation of arginase activity in crude tissue preparations have been inadequate in one or more respects. An improved procedure which has recently been developed (2) was employed in the present study.

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MATERIALS AND METHODS

Epidermis almost free of dermis was removed rapidly by scraping at room temperature (8) for the determination of arginase activity. The tissue was chilled in a weighing bottle in an ice bath immediately after removal, weighed rapidly on a torsion balance, and homogenized in a ground-glass homogenizer in ice-cold redistilled water. An aliquot of each homogenate was employed for the determination of total nitrogen by a semi-micro Kjeldahl procedure. Arginase activity was determined in aliquots of the freshly prepared homogenate in the presence and absence of added Mn^{++} and in samples incubated in 0.05 M $MnCl_2$ for 5 hours at 50° C. in the manner previously described (2). Only the results for activities measured in the presence of excess Mn^{++} before and after incubation will be reported because of the possibility of previous contamination of the epidermis with traces of activating ions. The results are expressed both on a fresh weight and on a total nitrogen basis. When the results were calculated in terms of dry weight of tissue the relative values differed little from those calculated in terms of nitrogen.

The samples of epidermis employed for the determination of total and TCA-soluble N were removed rapidly from the dermis at 50° C. on a constant temperature hot plate, weighed individually on a torsion balance, and then immediately placed in the calibrated tubes of small homogenizers containing water redistilled from permanganate which had been placed previously in gently boiling water. The tubes were removed after 10 minutes, cooled, the volume adjusted so that the quantity of fresh tissue was approximately 50 mg. per ml. and the tissue was homogenized. One ml. of the homogenate was employed for the determination of total nitrogen. To 2 ml. of the homogenate was added 0.5 ml. of 12.5 per cent TCA with stirring and the mixture was allowed to stand for 20 minutes. Nitrogen determinations were performed on aliquots of the filtrate. The filtrate gave no visible precipitate when more TCA was added or when picric acid, metaphosphoric acid, or a mixture of sul-

furic and phosphoric acids were added. Saturation with ammonium sulfate also had no effect. It is recognized that all known procedures for the precipitation of proteins from such complex mixtures give only comparative results. Nevertheless, when the conditions are carefully controlled valuable information can be gained about the relative qualities of nitrogen contained in higher and lower molecular weight fractions. The methods for the determination of urea and ammonia have been described previously (1).

Adult female Swiss mice were painted with methylcholanthrene in reagent grade benzene (0.6 gm. in 88 gm.), with 0.1 per cent croton oil in benzene, or with benzene alone. With one exception the experimental mice were sacrificed 5 days after the last application of the test material, and the control mice 5 days after the backs were shaved. The solutions were applied thrice weekly. The tumors studied were obtained from 2 lines of transplantable squamous cell carcinomata originally derived from primary tumors produced by the application of methylcholanthrene to the skin of a mouse. The analyses were limited to very small young tumors from which the connective tissue capsule and the small amount of central necrotic material were removed.

RESULTS

Arginase Activity in Normal Epidermis, Carcinogen-treated Epidermis, and Tumors—Normal epidermis had only slight arginase activity prior to heating with Mn^{++} (Table 1). However, after activating the preparations to the maximal extent under the conditions employed the mean value attained was 9 times greater than that found prior to activation. The arginase activity rose markedly after 3 paintings with methylcholanthrene in benzene so that even before activation the mean activity was greater than that of the normal epidermis after activation. The completely activated homogenate was approximately 3 times as active as the comparably treated normal tissue. After 11 paintings with the carcinogen the arginase level was considerably lower than after 3 paintings but was still slightly higher than normal. However, when 24 applications were made over a period of 8 weeks the arginase activity was reduced to slightly lower values than were found in the normal controls. Maximally activated homogenates of Tumors I and II were approximately 6 and 18 times more active than normal epidermis on a fresh weight basis and approximately 11 and 33 times more active when expressed in terms of total nitrogen. These values were also considerably higher than those found after 3 paintings. The arginase activity of the tumors was relatively greater when the results were expressed on the nitrogen basis than when wet weights were employed because a given wet weight of tumor tissue contains only approximately one-half of the dry weight (8) or nitrogen (see Table 3) found in an equal fresh weight of normal epidermis.

The results show clearly that the maximal potential arginase activity of the transplantable squamous cell carcinomata is much greater than that found in the other tissues of the carcinogenic series. The relative increase in activity on incubation at 50° C. with Mn^{++} was greater for Tumor II than for any of the other tissues examined while that for epidermis receiving 3 paintings was the smallest. The physiological significance of this latter finding is not yet clear. With the exception of liver, Tumor II exhibited the highest arginase activity of any tissue studied to date by the method of determination employed (2).

Influence of Benzene Alone and Benzene Containing 0.1 Per Cent Croton Oil on Arginase Activity—Six and 11 paintings with benzene alone produced approximately a two fold increase in the arginase activity of epidermis (Table 2). When benzene containing 0.1 per cent croton oil was applied 6 times over a period of 2 weeks the arginase activity was normal. The hyperplasia which the latter solution produces, which is somewhat greater than that resulting from benzene alone, regresses after cessation of treatment and does not result in tumor formation.[1] When the croton oil solution was applied daily for 6 days and the animals were killed on the day following the last treatment an even greater hyperplasia was found on histological examination than in the previous 2 groups. However, in the latter tissue the arginase activity was lower than that found in the normal epidermis. These results indicate that the stimulation to growth of the epidermis by a non-carcinogenic agent does not necessarily result in an increase in the arginase activity of the tissue. Epidermis which had received 11 paintings with benzene had greater arginase activity and less hyperplasia than that shown by tissues receiving the same number of applications of benzene containing the carcinogen.

Total Nitrogen, Tissue Weights, and TCA-soluble Nitrogen in Normal Epidermis and in Carcinogenesis—The weights of epidermis per mouse were determined on samples obtained by heat from mice in which approximately equal areas were shaved, painted, and removed. It should be noted that in the tissue receiving 24 paintings with the methylcholanthrene solution there are projections of epidermis into the dermis. An undetermined portion of this material was left behind in the dermis when the separation was made. The area of epidermis used may also have been slightly smaller in the 24 paintings material.

[1] Berenblum, personal communication, and unpublished observations in this laboratory.

TABLE 1

Arginase Activity in Epidermal Carcinogenesis

(Results are expressed in micrograms of urea liberated in the presence of Mn^{++} under standard assay conditions)

Tissue	Number of samples	Number of mice	Arginase activity: Before activation — Micrograms per mg. fresh wt.	Arginase activity: Before activation — Micrograms per mg. total N	Arginase activity: After activation — Micrograms per mg. fresh wt.	Arginase activity: After activation — Micrograms per mg. total N
Normal	8	40	3§ (1–10)‖	73 (30–110)	27 (19–37)	586 (421–830)
3 paintings with MC*	10	30	37 (24–46)	615 (448–842)	83 (57–121)	1,540 (1,120–2,190)
11 paintings with MC	5	15	10 (5–18)	179 (97–329)	38 (31–59)	675 (536–1,090)
24 paintings with MC	10	38	2 (0–4)	43 (0–88)	20 (16–25)	421 (328–495)
Tumor I†	10	10	24 (10–68)	995 (431–2,700)	165 (64–257)	6,690 (2,980–9,360)
Tumor II‡	10	10	39 (8–71)	1,579 (330–2,500)	479 (160–721)	19,400 (6,620–27,700)

* Methylcholanthrene in benzene
† 58th transplantation
‡ 6th transplantation
§ Average value
‖ Range of values

TABLE 2

Influence of Pure Benzene and Benzene Containing 0.1 per cent Croton Oil on Arginase Activity

(Results are expressed in micrograms of urea liberated in the presence of Mn^{++} under standard assay conditions)

Tissue	Number of samples	Number of mice	Arginase activity: Before activation — Micrograms per mg. fresh wt.	Arginase activity: Before activation — Micrograms per mg. total N	Arginase activity: After activation — Micrograms per mg. fresh wt.	Arginase activity: After activation — Micrograms per mg. total N
Normal	8	40	3 (1–10)	73 (30–110)	27 (19–37)	586 (421–830)
6 paintings with benzene	6	20	7 (6–11)	155 (121–192)	58 (48–65)	1,244 (1,090–1,400)
11 paintings with benzene	3	15	13 (5–21)	165 (107–199)	63 (40–81)	1,167 (795–1,385)
6 paintings with croton oil in benzene	6	11	5 (2–7)	112 (46–195)	28 (11–43)	598 (253–949)
6 paintings with croton oil in benzene*	6	15	6 (4–9)	112 (76–160)	18 (12–27)	347 (226–486)

* This group of animals was painted on 6 successive days and the epidermis was analyzed on the day after the last treatment. All other experimental groups were painted thrice weekly and killed 5 days after the last painting.

TABLE 3

Total Nitrogen and TCA-soluble Nitrogen in Epidermal Carcinogenesis

Tissue	Number of samples	Number of mice	Weight of epidermis: Mg. per mouse	Total nitrogen: Per cent of fresh wt.	TCA-soluble nitrogen: Per cent of fresh wt.	TCA-soluble nitrogen: Per cent of total N
Normal	7	30	56 (48–62)	4.56 (3.98–5.05)	0.79 (0.68–0.84)	17.4 (15.6–20.0)
3 paintings with MC*	12	29	127 (98–195)	5.39 (5.19–6.08)	0.74 (0.70–0.79)	13.7 (11.6–17.5)
6 paintings with MC	5	15	92 (71–98)	4.76 (4.64–5.00)	0.82 (0.69–0.92)	17.2 (14.8–19.9)
24 paintings with MC	10	32	59 (47–68)	4.66 (4.32–5.05)	0.95 (0.84–1.14)	20.3 (18.1–22.1)
Tumor I†	10	10		2.28 (1.97–2.61)	0.23 (0.17–0.28)	10.1 (7.7–11.7)

* Methylcholanthrene in benzene
† 62nd transplantation

The weights of the epidermal sample per mouse showed an increase to 127 mg. per mouse after 3 paintings from the normal mean value of 56 mg. (Table 3). The values dropped to 92 mg. and 59 mg., respectively, after 6 and 24 paintings with methylcholanthrene. The total nitrogen showed a pattern of changes similar to that found in the weights, increasing markedly after 3 paintings and decreasing thereafter. In contrast, the TCA-soluble N decreased after 3 paintings and increased thereafter. Both the total nitrogen and TCA-soluble N were lower in the tumor studied because of the much higher water content of the tissue. No comparison of the weights of the transplanted tumors and the epidermal samples is possible. The mean percentage of the total nitrogen found in the TCA-soluble fraction decreased from 17.4 in the untreated controls to 13.7 after 3 paintings of the carcinogen, returned to 17.2 per cent after 6 applications, and rose to 20.3 per cent after 24 applications. These changes were in the opposite directions from the changes in arginase activity, weights, and total nitrogen. In the tumor studied the percentage of TCA-soluble N was 10.1, a value lower than that found in any of the samples of epidermis. This is consistent with findings which showed that there is a marked decrease in the free amino acids in the tumors (6) and with the decrease in urea and ammonia to be reported in a subsequent section of this paper.

Influence of Benzene Alone and Benzene Containing 0.1 Per Cent Croton Oil on Total Nitrogen, Tissue Weights, and TCA-soluble N—Three applications of benzene alone produced a slight increase in the average weight of the epidermis, while after 6 treatments there was a slight decrease from the normal values (Table 4). There was a decrease in the total nitrogen content of these tissues and a concomitant increase in the TCA-soluble N. These changes resulted in a significant increase in the percentage of the total N found in the fraction not precipitated by TCA under our experimental conditions. However, in the tissues receiving 6 paintings with benzene containing croton oil there was an increase in the total nitrogen and a corresponding increase in the TCA-soluble N, the ratio of the two remaining in the normal range.

Urea and Ammonia in Epidermal Carcinogenesis—Three applications of methylcholanthrene in benzene produced a decrease of urea nitrogen from 77 mg. per cent to 44 mg. per cent (Table 5). No further significant decrease took place in tissues receiving 6 and 24 paintings of the carcinogen. There was also a fall to 48 mg. per cent in the epidermis of mice which had received methylcholanthrene contained in a mixture of benzene and lanolin (0.6 gm. methylcholanthrene in 88 grams of a mixture containing equal weights of lanolin and benzene). It has been shown that when lanolin alone is the solvent for methylcholanthrene the carcinogenic action is prevented, although the tissue is sensitized to further applications of the carcinogen contained in pure benzene (9). Methylcholanthrene contained in a mixture of benzene and lanolin also does not exert its customary action (10). The sebaceous glands are not destroyed and little if any hyperplasia occurs. It is, therefore, apparent that the decrease in urea observed in the animals receiving methylcholanthrene in benzene does not necessarily depend on the destruction of the sebaceous glands, which occurs after 3 paintings, or on the production of the intense hyperplasia which is usually induced by this treatment. The application of a dilute solution of croton oil in benzene, a non-carcinogenic agent, was previously reported to produce a decrease in epidermal urea (1) comparable to that produced by methylcholanthrene in benzene in the present study. It can, therefore, be concluded that agents other than carcinogens can induce a marked decrease in epidermal urea. No significant changes in the ammonia content of the treated tissues were observed with the possible exception of the decreased ammonia level in the tissue treated with

TABLE 4

INFLUENCE OF PURE BENZENE AND BENZENE CONTAINING 0.1 PER CENT CROTON OIL ON TOTAL NITROGEN AND TCA-SOLUBLE NITROGEN CONTENT

Tissues	Number of samples	Number of mice	Weight of epidermis Mg. per mouse	Total nitrogen Per cent of fresh wt.	TCA-soluble nitrogen Per cent of fresh wt.	TCA-soluble nitrogen Per cent of total N
Normal	7	30	56 (48–62)	4.56 (3.98–5.05)	0.79 (0.68–0.84)	17.4 (15.6–20.0)
3 paintings with benzene	6	22	67 (50–77)	4.19 (3.89–4.58)	1.06 (0.94–1.23)	24.8 (23.9–26.8)
6 paintings with benzene	5	20	43 (37–49)	4.19 (3.17–4.65)	0.92 (0.83–1.02)	24.3 (17.8–27.5)
6 paintings with croton oil in benzene	3	12	66 (46–73)	5.54 (5.40–5.63)	1.01 (1.00–1.01)	18.2 (17.7–18.7)

methylcholanthrene contained in the mixture of benzene and lanolin.

The mean values of the urea and ammonia contents of the tumors studied were significantly lower than those shown either by the hyperplastic or normal tissues. The samples obtained from the 57th transplantation gave closely similar mean values to those obtained from the 62nd transplantation of Tumor I. The values for Tumor II were slightly higher than those for Tumor I, but were, nevertheless, lower than those for any of the samples of epidermis studied.

DISCUSSION

The pattern of changes in arginase activity reported in the present study differs from that reported previously for cytochrome *c* (11), cytochrome oxidase (12), succinic dehydrogenase (12), and adenylpyrophosphatase (13). None of the other enzymes showed any significant changes from normal after 3 paintings with methylcholanthrene, while arginase increased three fold after this treatment. When the results were expressed on a wet weight basis, Tumor I had 6 times as much arginase activity as normal epidermis, while the succinic dehydrogenase and adenylpyrophosphatase activities increased two and three fold, respectively, and the cytochrome oxidase activity and the cytochrome *c* content were below the normal levels. In addition, pure benzene produced a doubling of the arginase activity while not exerting any influence on the other enzymes studied. There is some parallelism in the changes exhibited by arginase activity and those previously reported for cytoplasmic ribonucleic acid in similarly prepared material (14). On the other hand, the changes in desoxyribonucleic acid content were altogether different (15) and the patterns of changes in a number of other constituents studied to date (16) were not like those shown by the arginase activity.

The increased arginase activity after painting with benzene was accompanied by an increase over normal of the percentage of the total N found in the TCA-soluble fraction, while the elevation in arginase activity found in epidermis receiving 3 paintings of methylcholanthrene and the much greater increases in the carcinomata were accompanied by decreases in the proportion of TCA-soluble N. It is thus apparent that there is no obvious correlation between these variables which would hold for *all* of the tissues examined. However, in the *carcinogenic series* changes in arginase activity were consistently correlated with changes of opposite sign in the percentage of the total N accounted for by the TCA-soluble N. The samples of epidermis receiving 3 paintings with methylcholanthrene were more like the tumors with respect to arginase activity and TCA-soluble N than were any of the other samples of epidermis examined. The greatly increased epidermal weight and nitrogen content after 3 paintings suggest that the rate of protein synthesis is greater in this tissue than after a greater number of applications of the carcinogen.

The changes in urea level in carcinogenesis were not parallel to the changes in arginase activity. Even if arginase activity were entirely responsible for the urea content of epidermis, factors such as substrate concentration and cell permeability would play a role in the regulation of the tissue content of urea. Also if the ornithine cycle should be shown to exist in epidermis, arginase would only be one of a number of enzyme systems upon which the formation of urea would depend, and it is en-

TABLE 5

UREA AND AMMONIA IN EPIDERMAL CARCINOGENESIS

Tissue	Number of determinations	Number of mice	Urea-N	Ammonia-N
			Mg. per 100 gms. fresh tissue	
Normal epidermis	20	92	77 (55–98)	24 (16–31)
3 paintings with MC* in benzene	17	54	44 (32–66)	20 (16–28)
6 paintings with MC in benzene	5	15	42 (34–46)	28 (21–31)
6 paintings with MC in benzene + lanolin	5	25	48 (35–63)	17 (14–18)
24 paintings with MC in benzene	12	44	40 (26–67)	25 (20–31)
Tumor I†	10	10	21 (15–28)	7 (4–9)
Tumor I‡	10	10	22 (19–26)	9 (7–10)
Tumor II§	10	10	28 (20–46)	11 (9–15)

* Methylcholanthrene
† 57th transplantation
‡ 62nd transplantation
§ 5th transplantation

tirely possible that some of the other reactions would constitute the rate-limiting steps in the process. In this connection it is interesting to note that liver tumors have been shown to retain some of the arginase activity of normal liver but not the ability to convert ammonia to urea (17). The quantitative changes in urea in carcinogenesis resemble those reported for calcium (18) rather than those found in the nitrogen fractions reported in the present paper. On the other hand, the pattern of changes in ammonia in carcinogenesis was similar to that found for the TCA-soluble N. It is interesting that the mean values for urea and ammonia in tumors were closely similar to those previously found in mouse liver and in embryonic epidermis (1). The changes in urea level in carcinogenesis were negatively correlated with alterations in water content, since there was an increase in water content over normal in the hyperplastic tissues and a further increase (8) in tumors at the same time that progressive decreases in urea content were recorded.

Although the free arginine content fell from relatively high levels in normal and hyperplastic epidermis to values below the limits of detection in the tumors (6), the tumor tissue as a whole had the same content of arginine as normal epidermis (19). This indicates that the rapidly growing carcinoma is able to obtain arginine at a sufficiently rapid rate for protein synthesis in spite of the extremely low level of free arginine. The arginase activity of the carcinomata, which has been shown to be much greater than that of normal or hyperplastic epidermis in the present study, might be a factor limiting the quantity of arginine available for protein synthesis and thereby limiting the growth of the tumor.

SUMMARY

1. Determinations were made of arginase activity, total nitrogen, trichloroacetic acid soluble nitrogen, urea, and preformed ammonia in mouse epidermis treated with pure benzene or benzene containing croton oil or methylcholanthrene, and in transplantable squamous cell carcinomata originally derived from the skin of mice painted with methylcholanthrene solution.

2. All of the tissues studied showed a marked increase in arginase activity upon heating at 50° C. for 5 hours in 0.05 M $MnCl_2$.

3. The arginase activity, total nitrogen, and the wet weight of the epidermis per mouse all increased to a maximum after 3 paintings with benzene containing methylcholanthrene and decreased after subsequent applications of the carcinogen. The maximal mean value for arginase activity was 3 times that found in the untreated epidermis. The percentage of the total nitrogen found in the TCA-soluble fraction was lowest after 3 paintings.

4. In Tumors I and II the mean arginase activities were approximately 6 and 18 times, respectively, that of the normal epidermis on a wet weight basis and 11 and 33 times on a total nitrogen basis. The percentage of the total nitrogen found in the TCA-soluble fraction of the tumor samples studied was lower than in any of the samples of epidermis.

5. Six and 11 paintings of benzene alone produced a two fold increase in arginase activity, a decrease in total nitrogen content, and an increase in TCA-soluble nitrogen.

6. Three applications of methylcholanthrene in benzene caused a decrease in epidermal urea in adult mice to approximately 55 per cent of the normal value. No further decrease took place in tissues receiving 6 and 24 paintings of the carcinogen. No significant changes from normal were noted in the ammonia content of these tissues.

7. The level of urea decreased further in tumors to a value approximately one-third of that found in normal epidermis and the ammonia decreased to approximately the same extent.

8. The results were discussed with special reference to previous studies made on similarly prepared material.

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Tissue Culture Experiments on the Biological Action of Methyl *bis*(β-Chlorethyl) Amine and Its Hydrolysis Products

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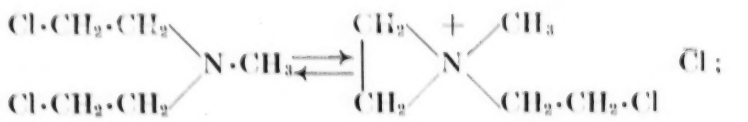
INTRODUCTION

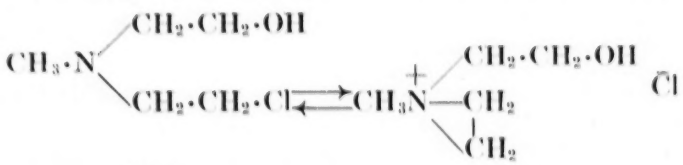
The β-chlorethylamines differ chemically from mustard gas and its analogues (the β-chlorethyl sulphides), whose effects on tissue culture were described in previous communications (5, 8), only in the replacement of a sulphur atom by an amino-radical. In many respects, the biological effects of the two classes of compound are similar, especially in their growth-inhibitory action on tissues which are in a state of active proliferation (6), and for this reason the "nitrogen mustards" in particular have already found clinical application, e.g. in the treatment of Hodgkin's disease. The present investigation concerns the action of methyl *bis*(β-chlorethyl)amine on fibroblasts cultivated *in vitro*. It was made during 1942 and 1943 on behalf of the Chemical Defence Research Department of the Ministry of Supply, and was originally described in two confidential reports (4). We are indebted to the Chief Scientist of the Ministry of Supply for permission to publish the results.

The experiments were made with the free base, (compound I). In aqueous solution, this undergoes a complex series of reactions. The first stage, achieved rapidly, involves cyclisation to the quaternary ethylenimonium chloride, (compound II):



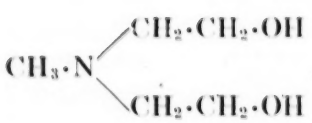
(I) (II)

A slower second stage yields either the mono- or di- hydroxy compounds, (III), (IV) or (V):

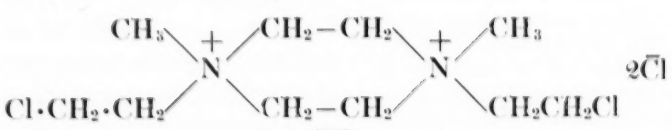


(III) (IV)

and



methyl diethanolamine, (V); or the piperazinium dimer:



(VI)

(Hornby et al, 7).

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The aqueous solutions of methyl *bis*(β-chlorethyl)amine develop an acute toxicity (as tested on mice), associated with a rapid convulsive and lethal action, and remain toxic indefinitely (3). In neutral solution, the maximum toxicity is reached after about two days. It has been attributed to the half-hydrolysis product, (III), or to the cyclised form, (IV), into which it is converted on standing. The dimer is not toxic. The effects on fibroblasts of the crude aqueous solutions of the base and of the individual hydrolysis products present in them are described in this paper.

MATERIAL AND METHODS

The tissue was obtained from the choroid and sclerotic of 11 to 12 day chick embryos and was grown by the ordinary hanging-drop method on 1¼ inch square coverslips; the culture medium was a mixture of equal parts of plasma, to which the active agents were added as described later, and of embryo extract made with Pannett and Compton's saline. The cultures were used for experiment after one or two passages in normal medium; for histological study they were fixed in 5 per cent acetic alcohol for 3 to 5 minutes, stained with Ehrlich's haematoxylin, and mounted whole in Canada balsam.

EXPERIMENTAL

I. *The effect of liquid chlorethylamine on cells in vitro.*—

The coverslips carrying the hanging-drop preparations were removed from the hollow ground slides and the cultures were placed in contact with drops of the liquid base on flat slides, the coverslips being supported on two strips of paraffin wax painted across the glass. The preparations were then re-sealed with wax and either observed on a microscope stage maintained at body temperature or returned to the incubator for various periods before being fixed and stained.

Immediately after the cells were in contact with the liquid, the fat globules in the cytoplasm began to swell rapidly, and in a few minutes the cells became heavily laden with refractile droplets precisely as in cultures treated with liquid mustard gas (5). Sometimes the infiltration was so complete that the globules fused with each other and the cells appeared as silvery, homogeneous bodies.

Apart from the infiltration of the protoplasm with droplets of the chlorethylamine, the cytological effects of this substance were very different from those of liquid mustard gas. Whereas the latter immediately coagulated the protoplasm, so "fixing" the cell with little distortion of either nucleus or cytoplasm, the liquid chlorethylamine caused both nucleus and cytoplasm to shrink continuously for some time after its application, and although a slow coagulation then began as indicated by the absence of Brownian movement inside the cell and a certain rigidity of its surface, the protoplasm never became opaque and refractile as in cultures treated with mustard gas. When the excess liquid was drained away, that infiltrating the cells disappeared and their structure could be examined in detail. They appeared greatly attenuated, their outlines were indistinct, and their nuclei extremely pycnotic, again in strong contrast to the cells of cultures treated with liquid mustard gas (Fig. 1).

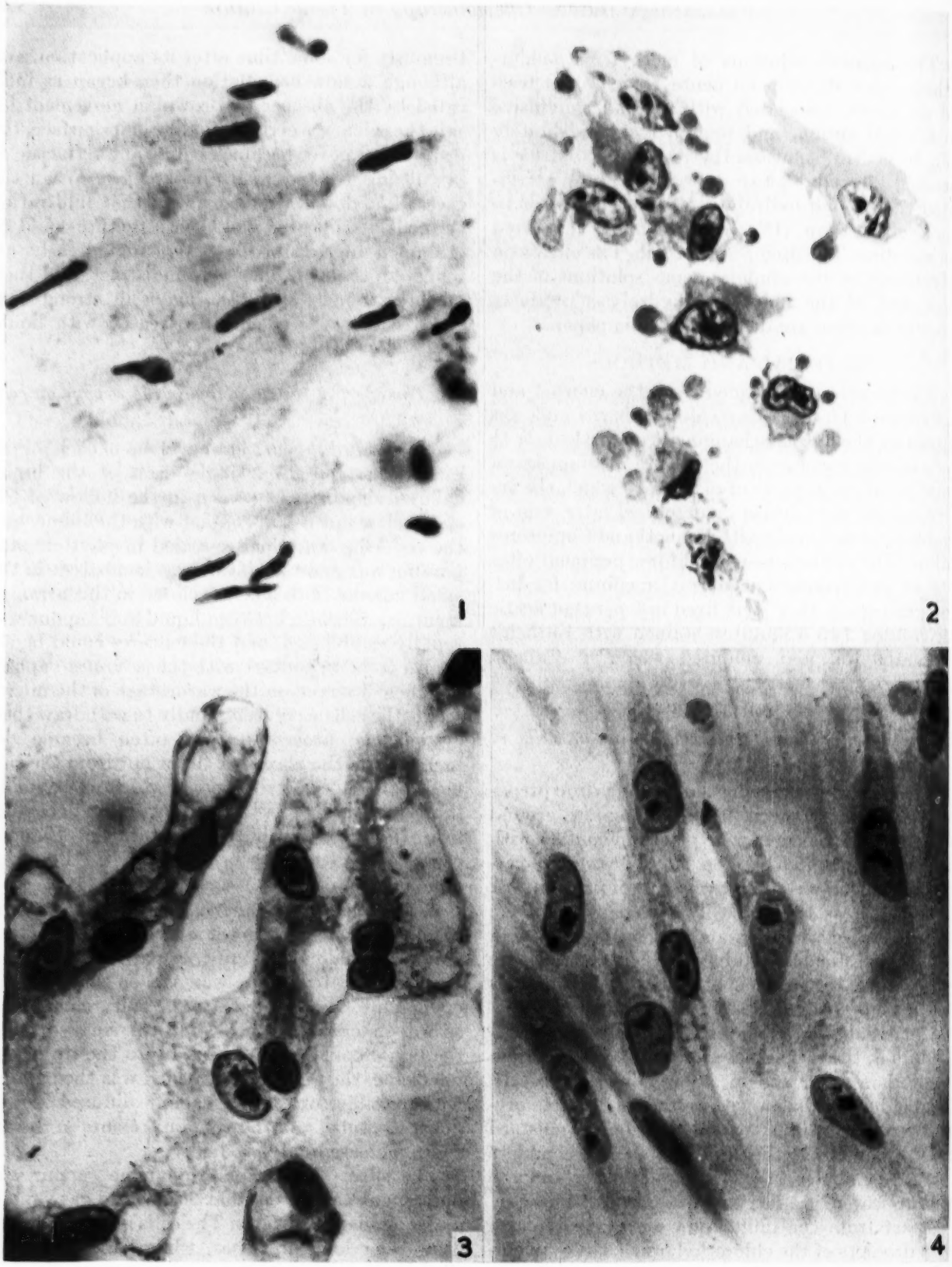
II. *The effect of the chlorethylamine vapour on cells in vitro.*—

1. *Saturated vapour.* The coverslip of each preparation was raised, a single drop of the liquid chlorethylamine was placed in the hollow of the slide below but not in contact with the tissue, and the coverslip was then re-sealed in position with paraffin wax and the culture was incubated. In the small volume (2 to 3 cc.) enclosed in this arrangement, equilibrium between liquid and vapour was rapidly established, and the cultures could be assumed to be in contact with the *saturated* vapour.

When observed on the warm stage of the microscope, the cells were seen rapidly to withdraw their cytoplasmic processes; they often became detached from the glass and threw out from the surface balloon-like protrusions which continually broke off, the cells disintegrating without coagulation (Fig. 2). Saturated mustard gas vapour causes little distortion because, like the liquid, it quickly coagulates or "fixes" the cells (5).

2. *Vapour at lower pressures.* For studying the cytological effect of lower concentrations of the vapour of chlorethylamine, a narrow inlet tube was incorporated in the wax seal so that one end opened into the air space of the culture chamber, as was described previously (5). A small drop of the liquid could be introduced into the tip of the inlet pipe, the outer end of which was then sealed with paraffin wax. The vapour diffused slowly down the tube, and the vapour pressure in the air space increased only gradually.

The effect of the chlorethylamine vapour was indistinguishable from that of mustard gas vapour under similar conditions. The cells gradually withdrew their long processes, while balloons of actively streaming cytoplasm were continually protruded and withdrawn at the surface. The shape of the cell and its nucleus became more and more distorted and fantastic, and small vacuoles often appeared in the interior; eventually the cells broke down.



All the cultures were fixed in acetic alcohol, stained with Ehrlich's haematoxylin and mounted whole. Magnification: ×875. (Photographed by Mr. V. C. Norfield).

FIG. 1.—Four-day culture of fibroblasts incubated in contact with liquid chlorethylamine for 30 mins., then fixed and stained. Note the pycnotic nuclei and the indefinite outline of the greatly attenuated cytoplasm.

FIG. 2.—Similar culture incubated in contact with saturated chlorethylamine vapour for 30 mins. before being fixed and stained. The cells are greatly deformed and beginning to disintegrate.

FIG. 3.—Culture of fibroblasts grown for 2 days in medium containing 200 γ/cc. of methyl diethanolamine. Note the shrunken nuclei and enormously vacuolated cytoplasm.

FIG. 4.—Normal (control) culture of fibroblasts from the same experiment as the preparation shown in Fig. 3.

III. *The effect on cells in vitro of low concentrations of methyl bis(β-chlorethyl)amine dissolved in the culture medium.* (*Table 1*).—

For these experiments, cultures were grown for two days in normal medium. They were then grouped into a number of equivalent sets of six and each set was transferred to medium containing a known concentration of chlorethylamine, one set being returned to normal medium as a control. After a further two days' incubation the cultures were fixed and stained. The descriptions in Table 1 refer to their condition at the end of this second incubation period.

TABLE 1

SUMMARY OF EXPERIMENTS SHOWING (A) THE EFFECT ON GROWTH OF DIFFERENT CONCENTRATIONS OF METHYL *bis*(β-CHLORETHYL)AMINE IN THE CULTURE MEDIUM, (B) THE DETOXICATING ACTION OF PLASMA ON THE CHLORETHYLAMINE.

No. of exp.	Concentration of chlorethylamine in medium (γ/cc)	Conditions of preparation of chlorethylamine solution	No. of cultures	Growth after 48 hours
1	0.0	Control	6	6: +++
	0.5	Added just before use	6	6: +++
	5.0	"	6	6: +++
	50.0	"	6	5: ++ 1: −
2	0.0	Control	6	6: +++
	50.0	Added just before use	6	2: + 3: ± 1: −
	50.0	Incubated 24 hours in plasma	6	6: ++
	100.0	Added just before use	6	1: + 1: ± 4: −
	100.0	Incubated 24 hours in plasma	6	6: ++
	200.0	Added just before use	6	6: −
	200.0	Incubated 24 hours in plasma	6	6: ++
3	0.0	Control	6	6: +++
	200.0	Added just before use	18	1: + 17: −
	200.0	Incubated 24 hours in plasma	18	12: +++ 6: ++

+++ = good growth; ++ = fair; + = poor; ± = very poor; − = no growth.

Experiment 1. The experimental culture media contained respectively 0.5, 5.0 and 50.0γ per cc. of chlorethylamine. They were prepared by vigorously stirring 0.01 cc. of the liquid with 10 cc. of fowl plasma at room temperature until it had dissolved. Portions of the solution were further diluted with appropriate volumes of plasma and immediately chilled at 0° C. The final clots to which the cultures were transferred were composed of equal volumes of embryo extract and of these plasma dilutions.

Table 1 shows that by the end of 2 days' incubation, a concentration of 50γ per cc. of the chlorethylamine had produced a slight but definite inhibiting effect on growth. This retardation of growth, however, was much more pronounced at the end of the *first* day, when 5 of the 6 cultures had no outgrowth at all, and the sixth very little. A sudden, surprising improvement occurred during the second 24 hours; the behaviour of the cultures suggested that the toxicity of the medium was diminishing, so that if a culture was not killed at the beginning of the experiment, it was subsequently able to revive and grow. It seemed that the culture medium itself might be exerting a detoxicating action, and this possibility was investigated in experiment 2.

Experiment 2. A solution of 0.01 cc. of the chlorethylamine in 10 cc. plasma was incubated for 24 hours at body temperature. Culture media were prepared from it, as described above, to contain respectively 50, 100 and 200γ per cc. of the base in the clot, and a set of cultures was transferred to each of these media. Three further sets were transferred to media containing the same concentrations of chlorethylamine, which had been prepared without the initial incubation. It was found (Table 1) that whereas a concentration of 200γ per cc. *prepared immediately before use* was lethal to the cells, the same concentration prepared from the chlorethylamine solution in plasma *which had been incubated for 24 hours*, had little effect on them.

This result was confirmed in *Experiment 3*, which was a repetition of experiment 2 at the high-

est concentration of the agent. Of 18 cultures transferred to freshly-prepared medium containing 200γ per cc. of chlorethylamine, only 1 showed slight growth; this exception may have been due to a slightly uneven distribution of the agent in the clot. On the other hand, 18 cultures in the same concentration of chlorethylamine prepared from a solution which was initially incubated for 24 hours, grew almost or quite as well as did the controls.

TABLE 2

EXPERIMENT SHOWING THE EFFECT ON THE DETOXICATION OF METHYL BIS(β-CHLORETHYL)AMINE BY PLASMA, OF VARYING THE PERIOD OF INCUBATION

No. of Exp.	Concentration of chlorethylamine in medium (γ/cc)	Incubation period of chlorethylamine in plasma	No. of cultures used	Growth after 48 hours
4	0.0	Control	6	6: +++
	200.0	2 hours	6	6: −
	,	6 ,	6	6: −
	,	12 ,	6	1: ++ 2: + 3: −
	,	24 ,	6	6: ++

It may therefore be concluded that: (1) the lethal concentration of "untreated" methyl *bis*(β-chlorethyl)amine for fibroblasts *in vitro* is between 100 and 200γ per cc.; (2) incubation in plasma diminishes the toxicity of this substance.

The sublethal concentrations of chlorethylamine, though not preventing cell growth, were not without effect, and the cultures grown in them displayed many abnormal mitotic figures. Clumping and fragmentation of chromosomes, multipolar figures and multinucleate cells derived from abnormal divisions were fairly common, though less abundant than in cultures grown in sublethal concentrations of mustard gas (5, 8).

IV. *Observations on the detoxicating action of plasma on methyl bis(β-chlorethyl)amine.*—

1. *Variation of the degree of detoxication with the time of incubation in plasma, Table 2, Experiment 4.* 0.01 cc. of liquid chlorethylamine was added to 5 cc. of plasma in each of 4 glass stoppered tubes, which were vigorously shaken and incubated at 37° C. for 2, 6, 12 and 24 hours respectively. Each solution was diluted with fresh plasma shortly before use so as to give a concentration in the culture clot of 200γ per cc. of free base, i.e. the lethal concentration when there has been *no* previous incubation. A set of 6 cultures was grown in each medium, and a control set in normal medium. The results are summarized in Table 2. Under the conditions of the experiment, there was no detoxication until after 12 hours' incubation in plasma; detoxication was not advanced until after 24 hours' incubation.

2. *The effect on detoxication of increasing the concentration of the chlorethylamine in the parent solution. Table 3. Experiment 5.* To 10 cc. of plasma in each of 4 tubes were added respectively 0.01 cc., 0.05 cc., 0.2 cc., and 0.5 cc. of liquid base. The tubes were thoroughly shaken and were then incubated for 24 hours. Each solution was diluted with fresh plasma immediately before use to give a concentration of 200γ per cc. in the final culture medium, and each was tested on a set of 6 cultures; a control set was kept in normal medium. The detoxicating action of the plasma, as can be seen from the table, decreased with increasing concentration of chlorethylamine.

V. *The effects on cells in vitro of "aged" aqueous solutions of methyl bis(β-chlorethyl)amine.*—

1. *Comparison of the influence on the toxicity of the chlorethylamine of incubation in (a) saline, (b) plasma. Table 4.* In *experiment 6*, 3 sets of cultures were grown in media containing 50, 100 and 200γ per cc. respectively of chlorethylamine which had been previously incubated at 37° C. for 24 hours in Pannett and Compton's saline; 3 other sets were grown in media containing the same concentrations of base which had been similarly incubated in plasma. The parent, incubated solutions each contained 0.025 cc. of chlorethylamine in 5 cc. of the "solvent"; after incubation the plasma solutions were diluted with fresh plasma and the saline solutions with embryo extract in such a way that all the culture clots ultimately contained the same proportion of embryo extract.

TABLE 3

EXPERIMENT SHOWING THE EFFECT ON THE DETOXICATION OF METHYL *bis*(β-CHLORETHYL)AMINE OF RAISING THE CONCENTRATION OF BASE IN THE PARENT SOLUTION. INCUBATION PERIOD: 24 HOURS.

No. of exp.	Concentration of chlorethylamine in medium (γ/cc)	Volume of chlorethylamine in 10 cc. of parent solution	No. of cultures used	Growth after 48 hours
5	0.0	Control	6	6: +++
	200.0	0.01 cc.	6	5: +++ 1: infected
	,	0.05 ,	6	6: ++
	,	0.20 ,	6	2: ± 4: −
	,	0.50 ,	6	2: + 4: −

Of the 18 cultures transplanted to medium containing the saline-incubated chlorethylamine, only 2, both in the lowest concentration, i.e. 50γ per cc., showed any growth. In contrast, all those grown in the media with plasma-incubated chlorethylamine grew except one in the 200γ per cc. concentration. The detoxicating action of the plasma in this experiment appeared to be rather less than in earlier ones.

2. *Increase in toxicity of the chlorethylamine after incubation in saline. Experiment 7. Table 4.* Sets of cultures were grown in media containing 50 and 100γ per cc. of the agent, which had been prepared from (a) fresh chlorethylamine added immediately before use, (b) chlorethylamine incubated in Pannett and Compton's saline as in experiment 6, and (c) chlorethylamine similarly incubated in plasma. The results set out in Table 4 show that at a concentration of 50γ per cc. the untreated base did not prevent, though it greatly restricted, the growth of the cultures, whereas the chlorethylamine which had been incubated in saline for 24 hours completely inhibited it, and the agent incubated in plasma was again largely detoxicated.

TABLE 4

SUMMARY OF EXPERIMENTS SHOWING (A) THAT THE DETOXICATING ACTION OF PLASMA ON METHYL *bis*(β-CHLORETHYL)AMINE IS NOT DIRECTLY RELATED TO HYDROLYSIS AND (B) THAT HYDROLYSIS INCREASES THE TOXICITY OF THE BASE.

No. of exp.	Concentration of chlorethylamine in medium (γ/cc.)	Treatment of chlorethylamine before being added to the culture medium	No. of cultures used	Growth after 48 hours
6	0.0	Control	6	6: +++
	50.0	24 hours' incubation in saline	6	2: ± 4: −
	50.0	" " " " plasma	6	6: ++
	100.0	" " " " saline	6	6: −
	100.0	" " " " plasma	6	6: +
	200.0	" " " " saline	6	6: −
	200.0	" " " " plasma	6	5: + 1: −
7	0.0	Control	6	6: +++
	50.0	Dissolved in saline shortly before use	6	2: + 4: ±
	50.0	24 hours' incubation in saline	6	6: −
	50.0	" " " " plasma	6	6: ++
	100.0	Dissolved in saline shortly before use	6	6: −
	100.0	24 hours' incubation in saline	6	6: −
	100.0	" " " " plasma	6	6: ++

3. *Detoxicating action of serum on hydrolysed chlorethylamine. Table 5.* For these experiments, serum was used instead of plasma because at the required concentrations the "aged" saline solutions of the chlorethylamine did not prevent the plasma from clotting during incubation. This substitution was controlled as described below, and did not influence the results. *Experiment 8:* The following solutions were prepared: (a) control: normal serum was incubated for 24 hours at 38.5° C; (b) 0.01 cc. of chlorethylamine was shaken with 5 cc. of serum and incubated for 24 hours; (c) 0.01 cc. of chlorethylamine was shaken with 5 cc. of plasma and incubated for 24 hours; (d) control: 0.1 cc. of chlorethylamine was shaken with 2.5 cc. of serum and incubated for 24 hours; another 2.5 cc. of serum was then added and the solution incubated for a further 24 hours; (e) control: 0.1 cc. of chlorethylamine was shaken with 2.5 cc. of Pannett and Compton's saline and incubated for 24 hours: an equal volume of saline was then added and the solution incubated for a further 24 hours; (f) 0.1 cc. of chlorethylamine was shaken with 2.5 cc. of saline and incubated for 24 hours; an equal volume of serum was then added and the solution incubated for a further 24 hours. All the solutions were diluted with fresh plasma immediately before use in such a way as to produce in the final culture media containing solutions (b) to (f) a concentration equivalent to 200γ per cc. of the free base, i.e. the concentration found to be lethal to cultures in the earlier experiments. A set of 6 cultures was transferred to each medium.

The results are set out in Table 5. Serum and plasma (solutions b and c) were equally effective in detoxicating the unhydrolysed chlorethylamine. Chlorethylamine hydrolysed in saline after 48 hours (solution e), at a concentration of 200γ per cc., killed all the cultures. In contrast, when it was initially incubated in saline for 24 hours and then treated for 24 hours with fresh plasma (solution f), it only partially inhibited growth.

This observation was confirmed in *experiment 9.* Solutions (i), (j) and (k), Table 5, were repetitions of solutions (d), (e) and (f) of experiment 8; but the two control solutions (g) and (h) were different. Solution (g) contained the same proportion of saline as did solution (j), and to solution (h) the same quantity of previously incubated serum was added as was present in solution (i). The experiments indicate clearly that the hydrolysis products of methyl *bis*(β-chlorethyl)amine can be partially detoxicated by the action of serum.

VI. *The toxicity of the substances produced during hydrolysis of methyl bis(β-chlorethyl)amine.*—

1. *Methyl diethanolamine* (compound V). The toxicity of the di-hydroxy-derivative of the chlorethylamine was compared with that of the parent chloro-compound in *experiment 10*. Fifteen mg. of the compound was dissolved in 1 cc. of plasma, and diluted with fresh plasma so as to give concentrations in the culture clot of 200, 1000 and 2000γ per cc., solutions (b), (c) and (e), table 6; the solutions were used shortly after preparation. Solutions (d) and (f), which contained the same concentrations of agent as solutions (c) and (e) respectively, were prepared from an initial solution of 22.1 mg. in 1 cc. plasma which had been incubated for 24 hours at 37° C. before being diluted with fresh plasma. One set of cultures was grown in each medium and a control set in normal medium.

TABLE 5

SUMMARY OF EXPERIMENTS SHOWING THAT HYDROLYSED METHYL *bis*(β-CHLORETHYL)AMINE CAN BE PARTLY DETOXICATED BY INCUBATION IN SERUM

No. of exp.	Concentration of chlorethylamine in medium (γ/cc.)	Solution added to the culture medium	No. of cultures used	Growth after 48 hours
8	0.0	(a) Normal serum incubated 24 hours	6	6: ++
	200.0	(b) Chlorethylamine incubated 24 hours in serum	6	6: +++
	"	(c) " " 24 " " plasma	6	6: +++
	"	(d) " " 48 " " serum	6	5: +++ 1: +
	"	(e) " " 48 " " saline	6	6: −
	"	(f) " " (a) 24 " " saline (b) 24 " " serum	6	6: +
9	0.0	(g) Normal saline	6	6: +++
	0.0	(h) Normal serum incubated 24 hours	6	6: +++
	200.0	(i) Chlorethylamine incubated 48 hours in serum	6	6: +++
	"	(j) " " 48 " " saline	6	6: −
		(k) " " (a) 24 " " saline (b) 24 " " serum	6	6: +

TABLE 6

EXPERIMENT SHOWING (A) THE EFFECT ON GROWTH OF DIFFERENT CONCENTRATIONS OF METHYL DIETHANOLAMINE AND (B) THAT METHYL DIETHANOLAMINE IS NOT DETOXICATED BY INCUBATION IN PLASMA.

No. of exp.	Concentration of diethanolamine in medium (γ/cc)	Treatment of diethanolamine	No. of cultures used	Growth after 48 hours
10	(a) 0.0	Control	6	6: +++
	(b) 200.0	Added just before use	6	6: ++
	(c) 1000.0	" " " "	6	6: +
	(d) 1000.0	Incubated 24 hours in plasma	6	6: +
	(e) 2000.0	Added just before use	6	6: −
	(f) 2000.0	Incubated 24 hours in plasma	6	6: −

It will be seen from Table 6 that the lethal concentration of methyl diethanolamine lies between 1000 and 2000γ per cc. as compared with 200γ per cc. of the chlorethylamine, i.e. it is less toxic. Under the conditions of the experiment, it was not detoxicated by incubation in plasma.

In all the concentrations used, methyl diethanolamine had a fairly drastic effect on the structure of the cells (cf. Figs. 3 and 4). The nuclei were rather shrunken and the cytoplasm of the fibroblasts in all the treated cultures became honeycombed with vacuoles; many of the cells grown at a concentration of 1000γ per cc. were reduced to a mere shell of protoplasm enclosing an enormous central vacuole. These vacuoles contained a clear fluid in which particles in active Brownian movement were seen. The formation of cytoplasmic aqueous vacuoles in fibroblasts treated with various nitrogen compounds has been described by other observers (9, 10).

2. *Methyl β-hydroxy-ethyl-β-chlorethylamine; compound III.* Experiments were made to determine the lethal concentration of the mono-hydroxy-derivative of the chlorethylamine. On account of its very hygroscopic nature, it proved impossible to prepare culture media in which exactly weighed quantities of the small order required were dissolved; for this and other technical reasons, direct comparisons on sister cultures, of the toxicity of this substance with that of the parent compound, were found to be unreliable and they will not therefore be described in detail. Sets of cultures were, however, transferred to media containing concentrations of the hydroxy-derivative of the order of 7, 13, 27, 50, 100, 200 and 400γ per cc. After 48 hours, the cultures in concentrations of up to 27γ per cc. showed no growth and those at 13γ per cc. were subnormal. The mono-hydroxy-derivative, therefore, is at least as toxic to cells *in vitro* as is

the parent chlorethylamine and it is probably more toxic than the latter material.

Examination of the cultures grown in a concentration of 13γ per cc. after 24 hours showed no significant outgrowth, and that of the explants in 7γ per cc. was very poor. Both these sets underwent a marked recovery during the second 24 hours' incubation, suggesting that this substance also is detoxicated by the plasma.

3. *NN'-di-β-chlorethyl-NN'-dimethyl piperazinium chloride (the piperazinium dimer, compound VI).* Addition of relatively high concentrations of the dimer to the culture medium had no effect on cultures grown in it. It appeared to be nontoxic.

DISCUSSION

The results described above show that there is a close parallel between the relative toxicities of methyl *bis*(β-chlorethyl)amine and the products of its interaction with water, for cells *in vitro* and when injected into mice (3). Thus the chlorethylamine and its mono-hydroxy-derivative are very toxic both to tissue cultures and to mice, whereas the di-hydroxy-derivative is much less injurious and the dimer is non-toxic in relatively high concentrations. The toxicity of the parent substance and of its solutions in saline to fibroblasts *in vitro* may therefore be attributed to the presence in the culture medium of the mono-hydroxy derivative. Both the chlorethylamine and the mono-hydroxy-compound lose some of their toxicity when kept in contact with plasma at body-temperature; this suggests that the chloro-base reacts with protein, probably after partial hydrolysis since detoxication is not marked until after 24 hours' incubation. Evidence will be adduced elsewhere that such a protein-chloroethylamine compound of low toxicity can be produced experimentally under appropriate conditions.

The action of the liquid agent and its saturated vapour is less drastic than that of mustard gas in the same forms, but more obvious cell damage is produced since the cells are not immediately "fixed" by the nitrogen mustard. With low concentrations of the vapour, the effects of the two agents are very similar. The lethal concentration of methyl *bis*(β-chlorethyl)amine for cells *in vitro* is rather less than that of ββ'-dichlordiethyl sulphide, since cultures show some outgrowth in a concentration of 200γ per cc. of the former substance while growth is completely inhibited in the same concentration of the latter.

In sublethal concentrations the chlorethylamine, like mustard gas, causes severe mitotic disturbances including clumping and fragmentation of the chromosomes, multipolar figures and multinucleate cells. As noted elsewhere (5), the abnormalities resemble those caused by carcinogenic compounds and by x-rays. Similar mitotic abnormalities have been observed by Bodenstein (2) in the ectoderm of amphibian embryos treated with chlorethylamine, and Auerbach and Robson (1) have shown that this agent has a strong mutagenic action on *Drosophila*.

SUMMARY

1. Liquid methyl *bis*(β-chlorethyl)amine, when applied directly to tissue cultures, infiltrates the cells *via* the fat droplets as does liquid mustard gas; but, unlike the latter substance, it distorts the cells, which become attentuated and their nuclei pycnotic; it does not immediately coagulate them.

2. Higher concentrations of the vapour produce rapid distortion of the cells, which disintegrate without coagulation; low concentrations, like those of mustard gas vapour, slowly produce even more fantastic distortions of the fibroblasts, leading to their complete breakdown.

3. When chlorethylamine is added to the culture medium, its lethal concentration for cells *in vitro* is rather less than 200γ per cc., but if the agent is incubated in plasma at body-temperature for 24 hours before being added to the cultures, it loses much of its toxicity at this concentration. At higher concentrations the detoxication by incubation in plasma is less complete.

4. Incubation of the chlorethylamine in saline increases its toxicity; this toxicity is partially destroyed by further incubation in serum.

5. 24 hours' incubation in plasma is necessary to complete the detoxication of low concentrations of the chlorethylamine; the detoxication may therefore proceed through the hydrolysis products.

6. The second hydrolysis product of methyl *bis*(β-chlorethyl)amine, methyl diethanolamine, is much less toxic to cells *in vitro* than is the parent compound; the "half-hydrolysis" product is at least as toxic and perhaps more so; the piperazinium dimer is non-toxic. Since all these substances are present in aqueous solutions of the free chlorethylamine base, the toxicity of the compound may be due to hydrolysis of the mono-hydroxy-derivative. This substance also appears to be partially detoxicated by contact with plasma.

7. Sublethal concentrations of methyl *bis*(β-chlorethyl)amine cause mitotic abnormalities similar to those produced by mustard gas, carcinogenic substances, and x-rays.

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Nodular Hyperplasia of Thyroid Glands Induced by Thiouracil

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The prolonged administration of antithyroid compounds provides an experimental means by which the activity of endogenous pituitary thyrotropic hormone may be studied. The mechanism of action of these agents supposedly involves the inhibition of thyroid hormone formation, thus removing the controlling activity of the thyroid hormone upon the thyrotropic hormone of the anterior hypophysis (1, 2, 13, 14, 27, 34, 35).

A number of agents have been found which induce diffuse and nodular hyperplasia of the thyroid gland in rats by this mechanism. This is important because spontaneous thyroid tumors are rare in small laboratory animals. Recently, however, van Dyke found cystic papillary tumors in 56 per cent of his rats provided they were permitted to reach an age of 800 to 900 days. He related the development of spontaneous tumors in old rats to the ultimobranchial tissue and indicated the significance of involuting changes in the thyroid parenchyma adjacent to the tumors (49).

Among the agents which have been reported to induce adenomatoid hyperplasia of the thyroid gland in the rat were brassica seeds (24), thiourea (41, 42) thiouracil (5, 32), and acetyl aminofluorene with allylthiourea (3, 4). It has recently been shown, however, that 2-acetaminofluorene by itself produced thyroid tumors (6). Pulmonary metastases from malignant thyroid tumors have been described after thiourea (42).

In mice of several inbred and hybrid strains, marked hyperplasia of the thyroid glands without nodule formation has been described following prolonged feeding of goitrogens (8, 9, 17). Even though distinct tumor nodules failed to develop in the thyroid glands, thyroid tissue was found within the lung substance by these observers. Apparently species differences exist with respect to tumor formation in the thyroid gland.

The data to be reported summarize observations on the effect of thiouracil upon the thyroid gland of albino male and female rats, a preliminary communication having been published previously (32).

MATERIAL AND METHODS

A total of 111 albino rats of the Stanford strain, 56 males and 55 females, were used in the experiments. They were placed on the experimental diet at an average body weight of 100 grams, which corresponds to an average age of 45 days for the male and 55 days for the female rat in this colony. The basic diet was ground Purina dog checkers to which thiouracil was added in a concentration of 100 mgs. per cent. The drug was blended with the diet in a mechanical mixer for 30 minutes. The rats were permitted unrestricted access to both food and tap water. Several experimental groups were inoculated intraperitoneally with a culture of the L_4 strain of pleura-pneumonia-like organisms. They and the respective control groups were a part of a separate investigation reported elsewhere (48).[1] No essential differences were noted in the response of the thyroid glands and hypophyses between the inoculated thiouracil treated animals and the rats treated with thiouracil alone, so the former were included in this report.

The thyroid and adrenal glands and the gonads were fixed in Bouin's solution, the hypophyses in Zenker's formol after weighing the organs on a torsion balance. Sections were stained with hematoxylin and eosin, and in addition azocarmine-aniline blue stain was used on sections of the hypophyses.

Statistical procedures: Preliminary calculations showed that the thyroid weight in relation to the body weight in our rats closely paralleled the data published by Remington and collaborators (45) who used a strain of rats of the same ancestry and on the same diet. Hence, his data on 180 rats were combined with our data on 95 normal rats. From the combined data, the constants for the predicted thyroid weight were calculated according to the equation proposed by Huxley: $\log y = \log b + k \log x$ (28). For convenience a nomogram was then

[1] I should like to express my appreciation to Dr. W. C. Kuzell of the Department of Pharmacology for the privilege of studying his animals.

set up from which the per cent deviation of the observed thyroid weight from the predicted thyroid weight was readily obtainable.[2]

The individual values for the per cent deviations were then subjected to the usual statistical methods and the means of all groups compared with one another (12). The analysis was indicated because a considerable variation in thyroid weight within the same group was apparent.

RESULTS AND DISCUSSION

Weight changes.—Thyroid gland: The weights of the thyroid glands with the respective standard deviations (S.D.) and the same weights calculated as per cent deviations from the predicted weight with the standard deviations are summarized in Table 1.

In males, the mean thyroid weight changed very little between 120 and 261 days of the experimental period, the values for P being consistently greater than 0.05. Hence, the difference between the means was not considered to be significant. However, a significant difference between the mean thyroid weights was found when the rats which had been on thiouracil for 261 and 312 days respectively were compared. The difference was minus 224.07 and the value for P was smaller than 0.01. It indicated that a significant decrease in mean thyroid weight had occurred, and the change in weight was emphasized by structural alterations to be described later.

A progressive increase in thyroid weights was apparent when the data obtained from the female rats were analyzed. Thus, comparison of the means of groups F and H (120 and 155 days) gave a value for P of 0.02, that of groups H and K (155 and 266 days) a value for P of 0.02, the difference between the means being significant in both cases. A group of rats corresponding to the 312 day period of the males was not run, and it is not known if a similar decrease would have resulted with further continuation of feeding.

Hypophysis: The mean weights of the hypophysis in mgs. per 100 gms. of body weight are included in the last column of Table 1. The data were treated by statistical methods as described for the thyroid gland.

Calculating the statistical constants for male rats, the animals in groups D were found to have the largest glands with a mean weight of 6.5 mgs. $\pm$ 1.22. Further continuation of feeding (group E) resulted in a decrease in pituitary weight to 5.00 mgs. $\pm$ 0.93. Comparison of the means between D and E gave a value for P smaller than 0.01, hence highly significant.

In the females, a progressive increase in mean weight of the hypophysis was apparent, a maximum being reached in the rats which had been on thiouracil longest. The mean of group K when compared with any of the other groups was found to be significantly larger, with values for P of less than 0.01. In addition, there was a significant and progressive increase in mean pituitary weight beginning with group H (155 days), values for P being smaller than 0.05 in the remaining two groups when compared with one another.

Summarizing the observations on the weight changes, an increase in the weight of the thyroid gland and hypophysis was apparent in female rats which was progressive during the period of observation. No significant changes were demonstrable in male rats over corresponding periods of experimentation. A diminution in the weight of both glands was apparent in those male rats which had received thiouracil for an additional period of 50 days.

Microscopic changes.—Thyroid gland: Many of the histologic changes were similar to those which have already been reported in animals after administration of antithyroid compounds. The principal response was a diffuse hyperplasia of the epithelial lining of the follicles accompanied by a nearly complete loss of colloid (Fig. 1). The individual epithelial cells were high columnar and usually possessed basally placed large vesicular nuclei. Hyperemia was severe. The parathyroid glands, usually situated at the lateral margins of the glands, were completely surrounded by thyroid tissue on occasions. The diffuse hyperplasia was nearly uniform in both sexes and in all groups which had received the experimental diet for as long as 261 and 266 days respectively. Thereafter, and coinciding with a decrease in thyroid weight, the degree of epithelial hyperplasia was more variable within the group and even within the same gland. Thus, the height of the epithelial cells was reduced in 8 of the 20 animals which had received thiouracil for 312 days. Patchy accumulation of colloid-like material was noted in the follicles of 11 of the 20 rats, and colloid was uniformly present throughout the glands in 6 more rats (Fig. 2).

In a little more than one third of all the animals, aggregates of tubules were found in the hyperplastic glands (Fig. 3). They were lined by a columnar epithelium which was lower than that lining the follicles in the adjacent portions of the gland. The tubules had definite lumina in which a little granular and homogenous material was seen.

[2] I should like to thank Dr. A. E. Lewis of this Department for his help in many of the calculations and the preparation of the nomogram.

The nuclei of the epithelial cells were more compact and smaller than in the remaining gland. Short epithelial buds projecting slightly into the lumen were not infrequent. Groups of tubules traversed the width of the gland occasionally, although they were more frequent at the periphery. No definite relationship between the foci of tubular proliferation and the hyperplastic nodules to be described was evident.

In 37 of the 111 rats, or 33 per cent, areas of nodular hyperplasia were found. The lesions occurred as solitary or multiple nodules, the largest number in a single animal being four. The incidence is summarized in Table 2, in which the data are broken down with respect to sex, the number of rats having nodules, and the total number of nodules in each group. Small collections of acini fairly common. The majority of the nodules as well as the largest, which measured nearly 5 mms. in greatest diameter, had this histologic appearance.

A little different type of nodule which was seen in several instances is illustrated in Fig. 8. Such nodules, which were not encapsulated though well defined from the adjacent thyroid parenchyma, were composed of closely packed epithelial cells with a strongly acidophilic cytoplasm. The nuclei were hyperchromatic and located at the base of the cells, which formed small acini, often with a barely visible lumen. They were separated from one another by greatly distended endothelial lined channels containing blood. The largest of these nodules measured a little more than 2 mms. in greatest diameter.

TABLE 1

WEIGHTS OF BODY, THYROID GLAND AND HYPOPHYSIS IN ALBINO RATS ON A DIET CONTAINING 0.1 PER CENT THIOURACIL

Exper. period				Av. body weight	Thyroid gland		Mg. hypophysis per 100 gms. body wt.
		No. of			Weight and S.D.	Mean deviation*	
days	Sex	rats	Symbol	gms.	mgs.	per cent	mgs.
120	M	6	A	169.3	65.8±16.15	345.17±117.1	5.72±1.24
169	M	10	B	192.1	93.9±39.23	454.50±201.1	5.96±1.02
233	M	11	C	218.2	95.6±35.07	431.64±206.0	5.28±1.04
261	M	9	D	165.7	93.9±34.48	560.67±250.8	6.50±1.22
312	M	20	E	216.9	79.3±23.32	336.60±143.0	5.00± .93
120	F	7	F	153.7	48.8± 5.55	260.43± 59.1	5.86± .64
142	F	10	G	162.5	54.7±19.09	302.11±100.6	6.18±1.14
155	F	10	H	147.3	58.5±11.83	344.78± 84.2	6.28± .88
233	F	13	I	173.3	85.3±35.44	476.00±283.4	7.37±1.30
266	F	15	K	145.9	81.5±31.59	515.33±201.1	9.40±1.35

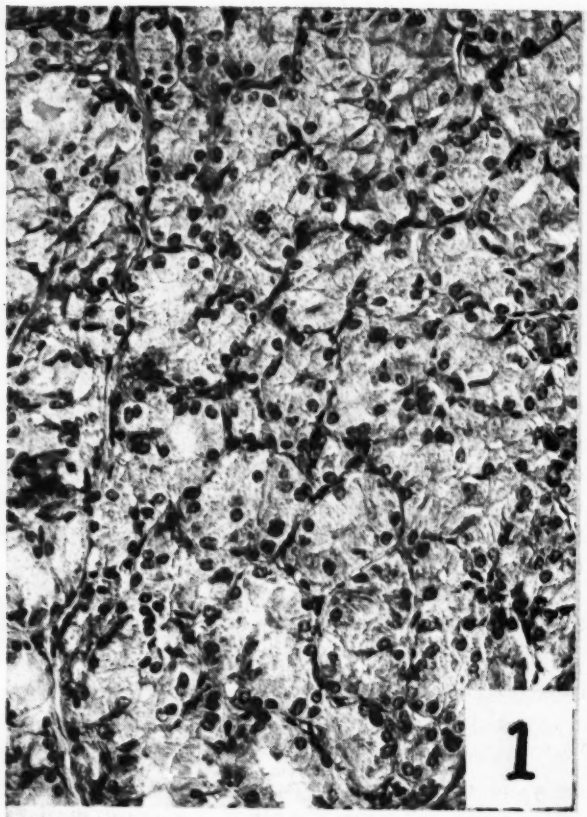
* Mean of per cent deviation of observed weight from predicted weight.

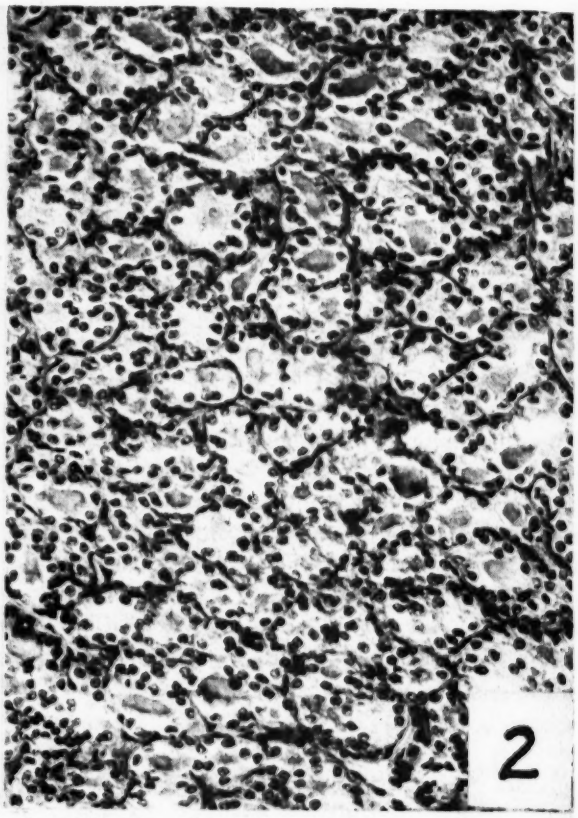
lined by epithelial cells with hyperchromatic nuclei were frequently encountered and thought to represent potential foci for the development of nodules (Fig. 4). They were not included in the table. There was some variation in the degree of epithelial proliferation, and examples are shown in the figures 5 to 8. In several nodules, the general arrangement of the acini closely resembled that seen in the adjacent thyroid tissue, from which they were separated occasionally by a little fibrous tissue (Fig. 5). The nuclei of the epithelial cells were more compact, however, and the nodules were readily visible by these two features. They measured up to 3 mms. in greatest diameter.

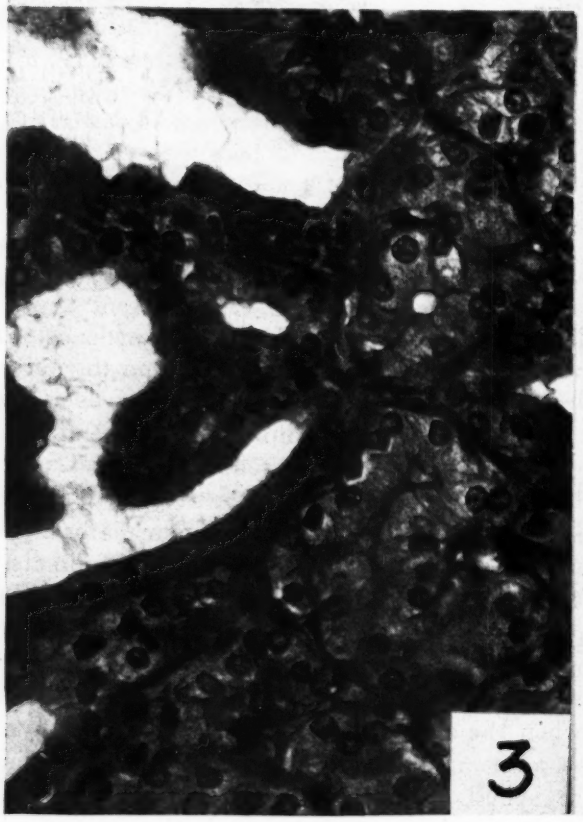
Other nodules were composed of cuboidal to columnar epithelial cells forming irregular acini which varied considerably in diameter and frequently contained vacuolated colloid-like material (Fig. 7). The nuclei, though uniformly found at the base, were hyperchromatic. Fibrous tissue was sometimes seen over portions of the periphery, yet the nodules were not encapsulated and prolongations into the adjacent thyroid parenchyma were

No obvious explanation has been found for the variability in the appearance of the nodules. It was not unusual to find two different types of lesions in the same gland. Definite evidence of invasiveness was not demonstrated in any of the lesions and they were considered to be benign.

Because a relationship between the development of spontaneous thyroid tumors and ultimobranchial cysts (49) had been suggested, a search for these structures was made in our material. The ultimo-branchial cyst was found in 14 animals, or 12.6 per cent, and in one of the animals each lobe contained the structure. The incidence is probably not a true one because serial sections were not made in the majority of the glands. However, when the animals bearing nodules in the thyroid were compared with those containing ultimobranchial cysts, it was found that only 2 rats had both nodules and cysts. In both instances, the cysts were widely separated from the nodules. The remaining 12 rats which had cysts, had no nodules. This suggested that the experimentally produced







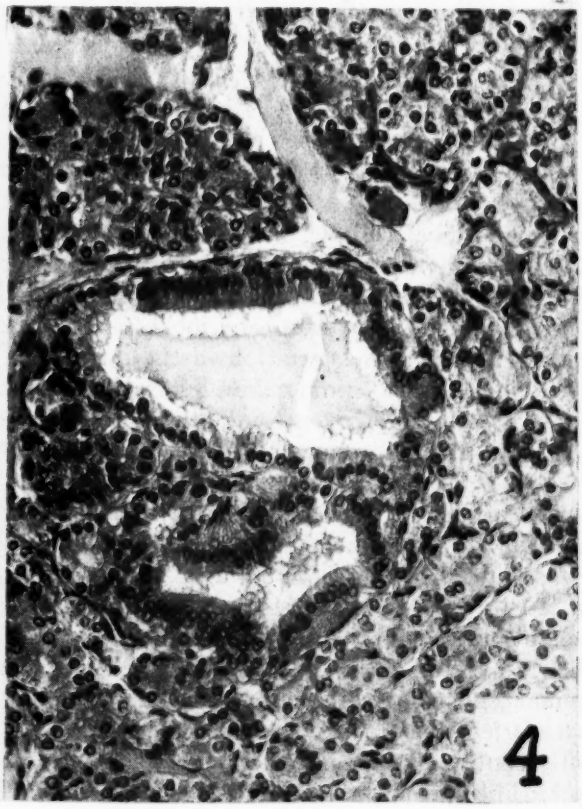


FIG. 1.—Diffuse hyperplasia of thyroid gland after 266 days of feeding thiouracil. Mag. ×220.

FIG. 2.—Accumulation of colloid in thyroid gland after 312 days of feeding thiouracil. Mag. ×220.

FIG. 3.—Area of proliferating tubules in hyperplastic thyroid after 142 days of feeding thiouracil. Mag. ×220.

FIG. 4.—Early focus of nodular hyperplasia after 266 days of feeding of thiouracil. Mag. ×220.

nodular hyperplasia occurring in young rats had little relationship to ultimo-branchial tissues.

Inflammatory reactions in the thyroid gland were fairly frequent but usually involved only the periphery, being most marked in the capsular fibrous tissue on the medial and posterior aspects of the glands. Among the cellular components, polymorphonuclear leucocytes with acidophilic granules, lymphocytes, and plasma cells were seen. No evidence of local vascular disease was found after special staining procedures for elastic and collagenous fibers. Further investigations supported the impression that the inflammatory reaction was not directly related to the effects of the drug because various degrees of inflammation of the tracheal mucosa and esophagus, with extension of the inflammatory reaction have been seen frequently in rats used for other purposes. Collections of lymphocytes such as are commonly seen in the human thyroid gland in Graves' disease were never encountered.

The significance of the change in granulation of the anterior lobe cells is difficult to interpret. It seems important, however, that the cytologic changes in the hypophysis coincided with the alterations in the weight and cytology of the thyroid gland, suggesting that they had a common underlying mechanism.

Summarizing the morphologic changes in the thyroid glands, diffuse hyperplasia of the thyroid gland was noted in animals which had been on thiouracil for periods varying from 120 to 260 days. Thereafter involution of the hyperplastic glands was noted, and it was associated with accumulation of secretion within the lumina of the acini. The thyroid glands from about one-third of all animals contained areas of nodular hyperplasia, and the incidence increased somewhat as the period of feeding was prolonged. Foci of tubular proliferation were noted in a little more than one-third of the animals but no relationship between these foci of tubular hyperplasia and the development of nodules was evident.

TABLE 2

INCIDENCE OF NODULAR HYPERPLASIA IN RATS ON THIOURACIL

MALES				FEMALES			
Exper. Period	No. of Rats	Nodular hyperplasia		Exper. period	No. of rats	Nodular hyperplasia	
		No. rats	No. nodules			No. rats	No. nodules
120	6	0	0	120	7	2	2
169	10	3	3	142	10	3	4
233	11	3	5	155	10	1	1
261	9	3	3	233	13	6	10
312	20	11	19	266	15	5	9

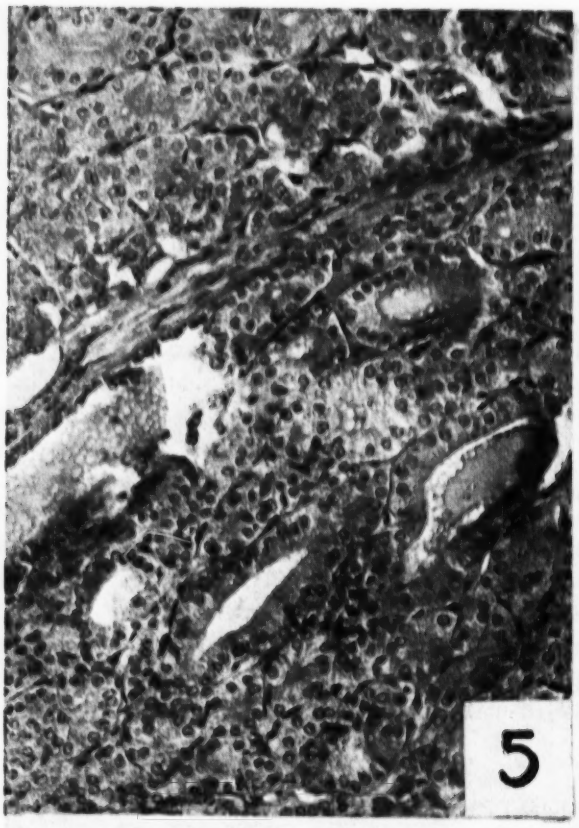
Hypophysis: Little emphasis was placed upon detailed cytologic examination of the anterior lobe cells in the present study. The majority of the glands were prepared for assaying procedures, and the results of this study are being published separately. Those glands which were available for histologic observations showed various stages of transformation of granulated basophiles to thyroidectomy cells and a marked diminution in granulated acidophiles. The changes which have been described by many observers (13, 21, 23, 30, 33, 36) were seen fairly consistently in the hypophyses. The male and female rats which had been on the diet for 261 and 266 days respectively and the males which had thiouracil for 312 days, however, presented a different cytologic picture. Five hypophyses were available in each of these groups. They contained granulated acidophiles. In contrast to the other groups, after 260 days, they were still infrequent and widely separated, and the granules were scant. After 312 days, numerous well granulated acidophiles were present and the number of thyroidectomy cells was smaller, granulated basophiles being now more prominent.

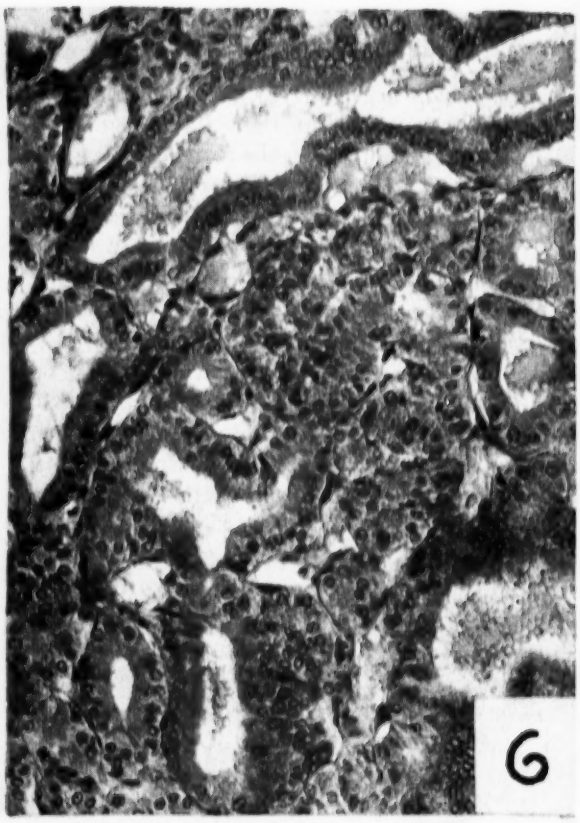
The hypophyses contained many thyroidectomy cells and there were only occasional poorly granulated acidophiles between 120 and 260 days of the experimental period. Thereafter, well granulated acidophiles occurred in large numbers and granulated basophiles were more frequently seen than thyroidectomy cells.

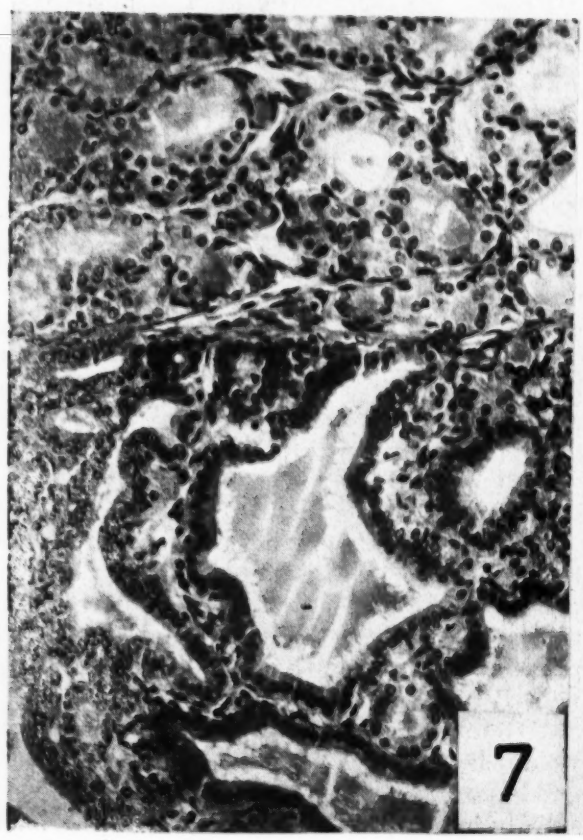
COMMENT

There is little doubt that the anterior pituitary thyrotropic hormone is responsible for the hyperplastic changes in the thyroid gland after administration of antithyroid compounds because hyperplasia is abolished in the hypophysectomized animal (1, 35, 26).

Determinations of thyrotropic hormone which would elucidate quantitative changes in the blood and hypophyses, have not yet yielded uniform results in hypothyroid states. While thyroidectomy is followed usually by an increase in the hormone in serum and hypophyses, the hypothyroidism produced by antithyroid drugs has given more variable results. It seems quite possible that the







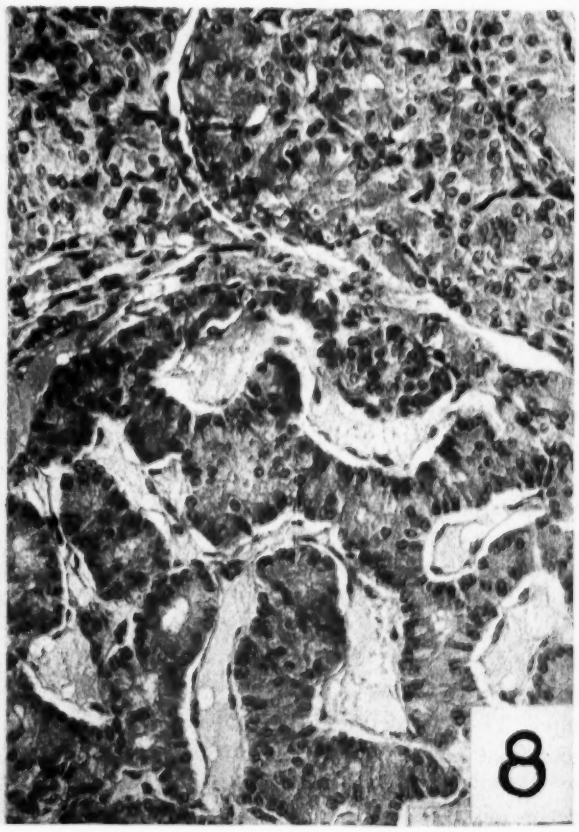


FIG. 5.—Border of thyroid nodule resembling adjacent thyroid parenchyma. Condensation of fibrous tissue at margin. 312 days of feeding. Mag. ×220.

FIG. 6.—Central portion of nodule shown in Figure 5. Mag. ×220.

FIG. 7.—Margin of thyroid nodule after 266 days of feeding thiouracil. Nuclei within nodule more hyperchromatic and epithelial height lower than in adjacent thyroid tissue. Mag. ×220.

FIG. 8.—Margin of nodule after 266 days of feeding thiouracil composed of a vascular network and cords and small follicles of epithelial cells. Mag. ×220.

removal of thyrotropic hormone from the circulation occurs in the presence of the thyroid gland, as has been suggested by Gordon and his collaborators (19). This mechanism does not exist in the thyroidectomized animal and the hormone may appear in detectable amounts in blood and urine.

Whatever the ultimate explanation of the quantitative changes in the production, release, and utilization of the thyrotropic hormone may be, our observations, and those of others, indicate that the thyrotropic hormone reaches the thyroid gland long enough and in such a quantity that active thyroid hyperplasia persists. Thus, our results show that the hyperplasia was maintained for about one-third of the life span of the experimental animal.

Diffuse hyperplasia is the earliest and most uniform response to antithyroid agents. Nodular hyperplasia may develop later in thyroid glands which still show diffuse hyperplasia in the remaining portions. In this respect, the experimentally induced thyroid nodules in young rats differ distinctly from the spontaneous thyroid tumors in old rats where involution of the thyroid parenchyma adjacent to the nodules has been described (49).

The observations on mice in which nodules do not develop in the thyroid gland even after prolonged exposure to these agents (8, 9, 17) are of importance. It seems reasonable that the cause for the development of nodules should be looked for in the thyroid gland itself. Since spontaneous tumors in that gland are rare in young rats, it may be suggested that the activity of the pituitary thyrotropic hormone provides the growth stimulus *per se*, and that the formation of nodules is one of the responses of which the thyroid is capable.

Although the nature of this particular mechanism is unknown, the focal or irregular type of response after periods of prolonged or, sometimes, exaggerated activity is not limited to the thyroid gland alone, and appears to be related in some way to age. In the hypophysis, for example, the incidence of nodular hyperplasia has been noted to be definitely increased in old rats (25, 40, 46, 51). Prolonged administrations of estrogens to rats, though first producing a diffuse type of cellular hyperplasia, have been found to be followed later by nodule formation in the hypophysis (11, 15, 16, 38, 47, 50, 52, 53). In man, the relation of aging to the incidence of nodular hyperplasia in hypophysis, thyroid glands and adrenals is frequently commented upon (7, 29). It is only possible, at this time, to point to the effects which aging or exaggerated stimulation may have upon the responses seen in various endocrine organs. It seems interesting to speculate on the possibility, however, that the chronic activity of growth stimulating substances upon endocrine organs may result in a state of reactivity in fairly young animals which, under normal circumstances, would have prevailed considerably later. That species differences may influence the responses, in addition, is possible and has been mentioned earlier.

While proliferative lesions in the thyroid gland are an undesirable side effect of the drug, Rawson and his collaborators (36) have shown that, in patients with Graves' disease treated with thiouracil, the hyperplasia of the thyroid gland can be restrained effectively by the simultaneous administration of iodine. Similar observations have been made in animals exposed to the combined activity of thiouracil and iodine for short periods of time (25, 32, 31). Further confirmation of the inhibitory effect of iodine upon thiouracil induced hyperplasia of the thyroid gland was obtained in our rats recently (16) where the animals had been exposed to both drugs for as long as 5 months. That the development of nodules may be prevented by this method is likely, although definite proof awaits continuation of such experiments for even longer periods than have been used thus far.

The cause or causes responsible for the structural and weight changes of the hypophyses and thyroid glands in rats which had been on thiouracil for 312 days were not evident. Attempts to demonstrate the mechanism should be made and include such possibilities as the development of a refractory state, the effect of aging, and exhaustion atrophy.

SUMMARY

The changes in weight and morphology of the thyroid gland and hypophysis following chronic administration of thiouracil in the diet have been described.

Analysis of the data has shown that a progressive increase in weight of both organs was limited to the female rats. There was evidence of involution of the thyroid gland in male rats which had received the diet for 10 months. The regressive changes in the thyroid gland were associated with cytologic alterations in the hypophyses, and these have been discussed briefly.

Diffuse hyperplasia of the thyroid gland was the most uniform response to the drug. In 33 per cent of all rats, definite nodular hyperplasia was found. The histologic structure of the nodules varied, and the same gland harbored nodules of different histologic appearance in several instances. All nodules were regarded as benign lesions.

Tubular proliferation of the epithelial cells lining the acini was seen in 40 per cent of the rats.

Examination of small foci of nodular hyperplasia failed to demonstrate a relationship between the tubules and the nodules, and both changes were seen in the same gland as distinctly separate structures.

Ultimo-branchial tissue was found in 12 per cent of the animals. Early lesions of nodular or tubular hyperplasia did not show a definite association with ultimo-branchial tissue such as has been found in spontaneous tumors of the thyroid gland in the rat.

Factors which may contribute to the development of nodular hyperplasia are discussed briefly in the light of comparable lesions in other endocrine organs and different species.

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New Books

A Symposium on the Use of Isotopes in Biology and Medicine: Addresses Delivered at an Institute Held at the University of Wisconsin from September 10 to September 13, 1947. Madison: University of Wisconsin Press, 1948. Pp. 445. Cloth.

This book contains twenty individual papers, contributed by experts in the field of biological application of isotopic technics. The articles are informative and cover a wide range of topics. The historical background is presented by H. T. Clarke (Columbia University). The separation, preparation, and availability of the isotopes are described, respectively, by H. C. Urey (University of Chicago), G. T. Seaborg (University of California), and P. C. Aebersold (United States Atomic Energy Commission). Applied aspects are contributed, among others, by H. G. Wood (Western Reserve University), K. Bloch (University of Chicago), and D. M. Greenberg and I. L. Chaikoff (University of California).

Interesting chapters by H. C. Urey on "International Aspects of Atomic Energy"; by W. F. Bale on "Health Hazards in the Use of Radioactive Isotopes"; and by F. Daniels on "Comments on the Development of Atomic Energy" are included.

The volume is readable and authoritative. It appears regrettably to have been published somewhat hurriedly by the photo-offset process and has no index. The latter would have been useful.

Sexual Endocrinology of Non-mammalian Vertebrates. By L. H. Bertschneider and J. J. Duyvené de Wit. ("Monographs on the Progress of Research in Holland during the War.") New York: Elsevier Pub. Co., Inc., 1947. Pp. 146. $2.75.

This monograph deals with research in the University of Utrecht during the war. Detailed morphological studies are presented of the pituitary and ovaries of a variety of nonmammalian vertebrates, with special emphasis on the bitterling. The purpose was to correlate structural and functional changes and to study the response to administration of various hormones.

These studies will be of special interest to biologists, physiologists, and those endocrinologists who may use fish in their research.

CORRECTION

The magnification for Figure 1 in the paper by Bern, Cancer Research **9**:68, February 1949, should read ×1.6. The magnification of Figure 2 on the same page should read ×24.